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<p>(54) Title: MODIFIED CELLS AND METHOD OF TREATMENT</p> <p>(57) Abstract</p> <p><i>Myc-induced cell death is inhibited in cultured cells, for example hybridoma cells, by expressing bcl2. Myc-induced cell death is de-inhibited in tumour cells by administering bcl2 antisense oligonucleotides. Compounds which modulate apoptosis may be detected in an assay using a proto-oncogene such as Myc.</i></p> <p>ATTORNEY DOCKET NUMBER: 10412-025 SERIAL NUMBER: 09/709,170 REFERENCE: A1</p>		



MODIFIED CELLS AND METHOD OF TREATMENT

This invention relates to genes involved in the regulation of programmed cell death (apoptosis) and modulation of the genes and their use in
5 prolonging the life of cells in culture and in methods of treatment of cancer and other diseases.

Human beings are large and long-lived organisms. They differentiate from a single progenitor cell into a complex multicellular organism and
10 maintain their architecture and organisation throughout adult life and in the face of continuous adventitious injury at both physical and genetic levels. One of the abiding paradoxes of human development is how individual cells in specific tissues know what they are, where they are, and whether or not to proliferate or differentiate. In part, they get this necessary
15 homeostatic information as short-range cues from their immediate neighbours in the form of cell-cell contacts and diffusible cytokines. However, these very localised signalling processes cannot easily explain the homeostasis and responsiveness of whole tissues. Recently, an appreciation has emerged of the role of innate cell death mechanisms in
20 the fashioning and establishment of complex somatic architecture. The principal form of programmed cell death is apoptosis.

Cancer is a disease caused by over-proliferation of individual clones of cells that arise in somatic tissues through mutation. The inappropriate and
25 uncontrolled growth that results disrupts the normal architecture of the organism and results in death by a variety of adventitious mechanisms. Because increased cell proliferation is the most prominent feature of cancer cells, cancer has almost exclusively been considered a disease in which the controls that determine the ability of an individual cell to
30 proliferate become damaged in some way. As such, almost all past

investigations of the molecular and biochemical mechanisms of cancer have concentrated on the machinery triggering cell proliferation (ie the dominant oncogenes) or suppressing it (the tumour suppressor or anti-oncogenes).

5

Apoptosis is the likely mechanism of cell death in a wide range of normal and pathological situations. Apoptosis is important during development in the establishment of complex architecture in limbs, the central nervous system, the immune system and many other tissues. Apoptosis has been
10 proposed as the mechanism of cell death of neurons in Alzheimer's and Parkinson's diseases and in CD4⁺ helper T cells in response to binding by HIV envelope glycoproteins during AIDS. In all of these latter pathological conditions, the disease arises because cell death occurs when it should not, in complete contrast to the situation in cancer when cell
15 death fails to occur when it should. Pharmacological intervention in cancer would focus on inducing apoptosis whereas in neurodegenerative and immunosuppresses pathologies intervention would seek to block death.

The *c-myc* gene is the cellular homologue of the viral oncogene *v-myc*
20 which is found in a number of avian and feline retroviruses that induce leukaemias and carcinomas. Recent evidence strongly suggests that the *c-myc* protein (Myc) is a transcription factor. It possesses a number of functional domains found in other proteins modulating transcription, specifically the leucine zipper characteristic of the FOS/JUN/CREB
25 transcription factor families and the basic-Helix-Loop-Helix motif found in, for example, the MyoD and E-box enhancer-binding proteins (Murre *et al*, 1989). Recently, both a heterodimeric partner and a consensus DNA-binding sequence for Myc have been identified. However, it is still unknown precisely which genes are regulated by Myc or to what
30 biological end.

The *c-myc* oncogene has been implicated in the control of normal cell proliferation by many studies. In particular, it is one of the immediate early growth response genes that are rapidly induced in quiescent cells upon mitogenic induction, suggesting that it plays some role in mediating the transition from quiescence to proliferation. However, unlike the majority of immediate early growth response genes, expression of *c-myc* is not confined to a brief period during the G_0/G_1 transition. Although a peak of *c-myc* expression in fibroblasts is observed some 3 hours after mitogenic stimulation, both *c-myc* mRNA and protein are continuously present at an appreciable level throughout the cell cycle in proliferating cells. As both *c-myc* mRNA and protein have very short half lives in fibroblasts (Waters *et al*, 1991), this sustained presence of Myc protein can only result from continuous synthesis. Ectopic induction of Myc activity is sufficient to drive quiescent growth factor-deprived fibroblasts into the cell cycle (Eilers *et al*, 1991). This argues that Myc regulates genes mediating the mitogenic response, an idea consistent with the protein's rapid induction by mitogens in quiescent cells. In addition, sustained expression of Myc can block both growth arrest and cell differentiation programmes, suggesting a role for Myc also in regulating genes mediating both of these processes.

Untransformed fibroblasts respond to serum or mitogen deprivation by growth arrest in a G_1 -like state often termed G_0 and can remain viable in this arrested state for extended periods. Mitogen withdrawal is accompanied by rapid down-regulation of *c-myc* expression at both the mRNA and protein level, irrespective of position within the cell cycle. Because cells deprived of growth factors eventually become quiescent it has been suggested that Myc down-regulation is a requirement or even a signal for growth arrest (Freytag, 1988; Waters *et al*, 1991).

In tumour cells, elevated or deregulated expression of *c-myc* (occasionally other *myc* genes) is so widespread as to suggest a critical role for *myc* gene activation in multi-stage carcinogenesis (Spencer and Groudine, 1991). Although it is unclear whether it is deregulation or over-expression of *c-myc* that comprises the major determinant in *c-myc* oncogene activation, it is nonetheless evident that *c-myc* activation disrupts the growth regulation of cells. For this reason, we have herein investigated the consequences of deregulated and elevated expression on the behaviour of rodent fibroblasts. In this report we show that deregulated *c-myc* expression is a potent inducer of programmed cell death (apoptosis) when combined with a block to cell proliferation.

In biotechnology, manipulation of programmed cell death is likely to have many uses. Many valuable cells used as sources of bio-reagents are difficult and expensive to maintain, requiring costly cytokines and careful maintenance. In addition, many cells favoured by biotechnology have a finite life-span *in vitro*. This is especially true in the case of cells used as sources of reagent destined for human use.

Primary human fibroblasts or epithelial cells are the commonest types of cell used. All such cells eventually senesce and die *in vitro*, often before they reproduce in sufficient yields for preparative purposes. Part of the process by which such cells fail in culture appears to be by execution of apoptosis. Thus, any means for abrogating apoptotic cell death is likely to be of significant benefit in both the propagation and the prolongation of mammalian cells culture.

Many cells used in culture are transformed, immortalized or derived from tumours. As a consequence, almost all have deregulated *myc* gene expression and are thus sensitised to processes that induce apoptosis. We

have theorised that this is precisely the reason why certain types of tumour cell die *in vitro* when subjected to nutrient privation (eg overgrowth, metabolite depletion) or exhaust growth factors that mediate their survival. A good example in this context is hybridomas - tumours derived by fusion
5 of lymphocytes with myeloma cells. Hybridomas require expensive growth media replete with multiple and costly growth factors or foetal calf serum, and even then are very prone to apoptotic death and poor cloning and growth, presumably due to transient and unintentional exposure to adverse growth conditions. We have now found that introduction of a
10 survival gene such as *bcl-2* into cultured cells potentiates their survival and renders them more resistant to the capriciousness of *in vitro* culture conditions.

The *bcl-2* proto-oncogene is the site on human chromosome 18 that is
15 reciprocally translocated to the Immunoglobulin Heavy Chain locus on chromosome 14 in a variety of B lymphoid tumours (Tsujimoto and Croce, 1986).

The *bcl-2* oncogene also synergises with the pervasive *c-myc* oncogene in
20 tumour progression in experiments where the two genes are introduced into transgenic mice and expressed specifically in B cells (Strasser *et al*, 1990). The mechanism of synergy was until recently unclear although the fact that Bcl-2 expression inhibits apoptosis (Hockenbery *et al*, 1990; Korsmeyer *et al*, 1990; Strasser *et al*, 1991) suggested that it might be this
25 property that was pertinent to its proto-oncogenic *modus operandi* (Korsmeyer *et al*, 1990). In the work described herein we have determined the mechanism of synergy between *c-myc* and *bcl-2* by demonstrating that the *c-myc* gene is a potent inducer of apoptosis, in addition to its established role in mitogenesis, and that expression of the
30 *bcl-2* gene specifically abrogates *c-myc*-induced apoptosis without affecting

the ability of *c-myc* to drive cell proliferation. This provides a novel mechanism for oncogene cooperation of potential importance both in carcinogenesis and in the evolution of drug resistance in tumours.

- 5 The findings disclosed herein, and particularly in the Examples, suggest that: (1) dominant oncogenes, specifically the pervasive oncogene *c-myc* and its homologues *N-myc* and *L-myc*, are potent inducers of cell death by apoptosis under conditions of withdrawal of survival cytokines or drug induced growth arrest; (2) that the proto-oncogenes *c-myc* and *bcl-2*
10 interact in a synergistic manner because *bcl-2* specifically abrogates the lethal effects of *c-myc* and leaves the mitogenic properties of *c-myc* unaffected; and (3) that expression of *bcl-2* renders cells significantly resistant to the cytotoxic effects of various drugs commonly used in cancer chemotherapy.

15

Thus, some of the objects of the invention are to make use of these findings in methods of treating tumour cells, methods of enhancing the life-span of, and yield of recombinant molecules from, cells in culture, and methods of assaying compounds for their apoptosis-modulating effects.

20

A first aspect of the invention provides a method of treating tumour cells in a vertebrate, the method comprising introducing into the tumour cells means for inhibiting an inhibitor of *myc*-induced programmed cell death.

- 25 Particular tumours suitable for treatment in accordance with the invention include leukaemias, and cancers of the uterine cervix, head, neck, brain glial cells, breast, colon, lung, prostate, skin, mouth, nose, oesophagus, stomach, liver, pancreas and metastatic forms of any of these.

- 30 It is preferred that the tumour cells to be treated are not lymphoma cells.

The means for inhibiting an inhibitor of *myc*-induced programmed cell death (apoptosis) may be provided by a means for preventing or reducing the expression of the said inhibitor in the said tumour cell, or it may be provided by a means for competing with the said inhibitor for the site of
5 action of the said inhibitor.

By "preventing or reducing the expression" we mean doing so to a useful extent such that *myc*-induced programmed cell death may proceed.

10 In one embodiment of the invention the said means for inhibiting comprises means for preventing expression of an anti-oncogene or proto-oncogene.

It is preferred if the anti-oncogene or proto-oncogene is *bcl-2* or a cellular
15 homologue thereof that fulfils the same function as *bcl-2*. Such a gene is likely to share at least 75% sequence identity with *bcl-2*.

Suitably, the expression of an anti-oncogene or proto-oncogene may be inhibited by the introduction into the cell of antisense nucleic acid adapted
20 to bind to the anti-oncogene or proto-oncogene or transcription products thereof.

Preventing or reducing *bcl-2* expression may be clinically useful in situations in which pathological hyperplasia arises in tissues that express
25 *bcl-2*, for example in the suppression of chronic or acute inflammation, and in the treatment of benign hyperplasias that lead to malignancy (for example as in familial polyposis coli in the gut).

It is preferred if the antisense nucleic acid is an antisense oligonucleotide.

Antisense oligonucleotides are single-stranded nucleic acid, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA-RNA, a DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that oligonucleotides could recognise sequences in the major groove of the DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesise sequence-specific molecules which specifically bind double-stranded DNA via recognition of major groove hydrogen binding sites.

By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A) addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

The antisense oligonucleotide can be used to selectively suppress certain cellular functions, for example the expression of the *bcl-2* protein.

Of course, the oligonucleotides may be "modified oligonucleotides".

By "modified oligonucleotides" we mean that they may contain phosphorothioate, methylphosphonate or other phosphoramidite internucleosidic linkages as well as, or instead of the usual phosphodiester linkages. Such internucleosidic linkages are less susceptible to nucleolytic degradation, or may confer on the antisense oligonucleotide other preferred pharmacokinetic properties. A further modification that can be

made instead of or in addition to the aforementioned modifications is the addition of a component capable of intercalating into the target nucleic acid, and thus stabilising the resultant (antisense oligonucleotide):(target nucleic acid) hybrid. The intercalating component is preferably acridine.

5

Although targeting of the oligonucleotide to a specific cell type is preferred, it is not necessary for the working of the invention because inhibition of *bcl-2* may not be deleterious to those cells which are not tumourigenic.

10

The antisense nucleic acid may additionally comprise a portion capable of targeting the antisense nucleic acid to cells generally or to a desired cell type.

15 By "capable of" we mean capable of targeting the antisense nucleic acid as said when the said targeting portion is joined to the antisense nucleic acid of the invention.

The targeting portion may specifically bind to a cell-type-specific entity or
20 may be specifically taken up by the specific cell type which is the intended target.

The entity recognised may be characteristic of cells in general, so that the antisense oligonucleotide is simply taken up into cells and is therefore
25 exposed less to extracellular nucleases, for example. The specificity of the compound is thus derived solely from the antisense oligonucleotide.

Alternatively, the entity which is recognised may be a suitable entity which is specifically expressed by tumour cells, virally-infected cells, cells
30 introduced as part of gene therapy or even specific normal cells of the

body into which, for whatever reason, one wishes to introduce the antisense oligonucleotide, but which entity is not expressed, or at least not with such frequency, in cells into which one does not wish to introduce the oligonucleotide. The entity which is recognised will often be an antigen. Examples of antigens include those listed in Table 1 below. A non-specific antigen is the transferrin receptor, to which antibodies may be raised, as taught in EP 226 419. Monoclonal antibodies which will bind specifically to many of these antigens are already known (for example those given in the Table) but in any case, with today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-specific portion may be an entire antibody (usually, for convenience and specificity, a monoclonal antibody), a part or parts thereof (for example an F_{ab} fragment, $F(ab')_2$, dab or "minimum recognition unit") or a synthetic antibody or part thereof. A compound comprising only part of an antibody may be advantageous by virtue of being less likely to undergo non-specific binding due to the F_c part. Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H. Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", J.G.R. Hurrell (CRC Press, 1982). All references mentioned in this specification are incorporated herein by reference. Bispecific antibodies may be prepared by cell fusion, by reassociation of monovalent fragments or by chemical cross-linking of whole antibodies, with one part of the resulting bispecific antibody being directed to the cell-specific antigen and the other to the oligonucleotide. The bispecific antibody can be administered bound to the oligonucleotide or it can be administered first, followed by the oligonucleotide. The former is preferred. Methods for preparing bispecific antibodies are disclosed in Corvalan *et al* (1987) *Cancer Immunol. Immunother.* 24, 127-132 and 133-137 and 138-143.

Bispecific antibodies, chimaeric antibodies and single chain antibodies are discussed generally by Williams in *Tibtech*, February 1988, Vol. 6, 36-42, Neuberger *et al* (8th *International Biotechnology Symposium*, 1988, Part 2, 792-799) and Tan and Morrison (*Adv. Drug Delivery Reviews* 2, 5 (1988), 129-142). Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies. IgG class antibodies are preferred.

Table 1

1. Tumour Associated Antigens

<u>Antigen</u>	<u>Antibody</u>	<u>Existing Uses</u>
5 Carcino-embryonic Antigen	{C46 (Amersham) {85A12 (Unipath)	Imaging & Therapy of colon/rectum tumours.
Placental Alkaline Phosphatase	H17E2 (ICRF, Travers & Bodmer	Imaging & Therapy of testicular and ovarian cancers.
Pan carcinoma	NR-LU-10 (NeoRx Corporation)	Imaging & Therapy of various carcinomas incl. small cell lung cancer.
10 Polymorphic Epithelial Mucin (Human milk fat globule)	HMFG1 (Taylor-Papadimitriou, ICRF	Imaging & Therapy of ovarian cancer, pleural effusions.
β -human Chorionic Gonadotropin	W14	Targeting of enzyme (CPG2) to human xenograft choriocarcinoma in nude mice. (Searle <i>et al</i> (1981) <i>Br. J. Cancer</i> 44, 137-144).
15 a Carbohydrate on Human Carcinomas	L6 (IgG2a) ¹	Targeting of alkaline phosphatase. (Senter <i>et al</i> (1988) <i>P.N.A.S.</i> 85, 4842-4846).
CD20 Antigen on B Lymphoma (normal and neoplastic)	1F5 (IgG2a) ²	Targeting of alkaline phosphatase. (Senter <i>et al</i> (1988) <i>P.N.A.S.</i> 85, 4842-4846).

¹Hellström *et al* (1986) *Cancer Res.* 46, 3917-3923

²Clarke *et al* (1985) *P.N.A.S.* 82, 1766-1770

Other antigens include alphafoetoprotein, Ca-125 and prostate specific
5 antigen.

2. Infectious Agent-Related Antigens

Mumps virus-related	Anti-mumps polyclonal antibody	Antibody conjugated to Diphtheria toxin for treatment of mumps.
10 Hepatitis B Surface Antigen	Anti HBs Ag	Immunotoxin against Hepatoma.

If applied to the treatment of CML or ALL, the ligand binding molecules can be monoclonal antibodies against leukaemia-associated antigens.
15 Examples of these are: anti-CALLA (common acute lymphoblastic leukaemia-associated antigen), J5, BA-3, RFB-1, BA-2, SJ-9A4 Du-ALL-1, anti-3-3, anti-3-40, SN1 and CALL2, described in Foon, K.A. *et al* 1986 *Blood* 68(1), 1-31, "Review: Immunologic Classification of Leukemia and Lymphoma". The ligand binding molecules can also be
20 antibodies that identify myeloid cell surface antigens, or antibodies that are reactive with B or T lymphocytes, respectively. Examples of such antibodies are those which identify human myeloid cell surface antigens or those which are reactive with human B or T lymphocytes as described in Foon, K.A. *Id.* Additional examples are antibodies B43, CD22 and
25 CD19 which are reactive with B lymphocytes can also be used.

Alternatively, the entity which is recognised may or may not be antigenic but can be recognised and selectively bound to in some other way. For

example, it may be a characteristic cell surface receptor such as the receptor for melanocyte-stimulating hormone (MSH) which is expressed in high numbers in melanoma cells. The cell-specific portion may then be a compound or part thereof which specifically binds to the entity in a non-immune sense, for example as a substrate or analogue thereof for a cell-surface enzyme or as a messenger. In the case of melanoma cells, the cell-specific portion may be MSH itself or a part thereof which binds to the MSH receptor. Such MSH peptides are disclosed in, for example, Al-Obeidi *et al* (1980) *J. Med. Chem.* 32, 174. The specificity may be indirect: a first cell-specific antibody may be administered, followed by a compound of the invention directed against the first antibody. Preferably, the entity which is recognised is not secreted to any relevant extent into body fluids, since otherwise the requisite specificity may not be achieved.

The targeting portion of the antisense nucleic acid of this embodiment of the invention may be linked to the remainder of the antisense nucleic acid by any of the conventional ways of linking compounds, for example by disulphide, amide or thioether bonds, such as those generally described in Goodchild, *supra* or in Connolly (1985) *Nucl. Acids Res.* 13(12), 4485-4502 or in PCT/US85/03312. A thiol group can be introduced at the 5'-end of an aminofunctionalised oligonucleotide (*Nucleic Acids Res.* (1991) 19, 4561). This group can be used to attach the oligonucleotide to a protein, such as a monoclonal antibody or growth factor, using standard heterobiofunctional protein cross-linking reagents such as *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS). These reagents usually link between a thiol group in one protein and the terminal amino group in a lysine residue in the other protein. Preferably, the linkage is cleavable in lysosomes by lysosomal enzymes or by the acidic environment to liberate the antisense oligonucleotide.

If desired, the antisense oligonucleotide can be conjugated with hydrophobic derivatives as taught in FR 2 649 321 to protect it from nucleases and to improve transport across cell membranes. The hydrophobic moiety may be cholesterol as taught by Zon in
5 "*Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*", pp 234-247, J.S. Cohen (Ed), CRC Press, Boca Raton, FL, 1989.

Conjugation of the oligonucleotides to poly-L-lysine may also enhance delivery of the said oligonucleotides to the cell as disclosed by Stevenson
10 and Iversen (1989) *J. Gen. Virol.* 70, 2673-2682, and by LeMaitre *et al* (1987) *Proc. Natl. Acad. Sci. USA* 84, 648-652.

In a similar fashion polyamines conjugated to phosphorothioate oligonucleotides enhance their cellular uptake as taught in US 5 138 045.
15

Suitable antisense oligonucleotides may be designed by reference to the sequence of the anti-oncogene or proto-oncogene. The sequence of the human *bcl2*-a cDNA (SEQ1) and its encoded amino acid sequence (SEQ2) are shown in Figure 13. It is preferable if the antisense oligonucleotide
20 hybridises to the region of the mRNA encoding the translational initiation codon, for example, in the case of *bcl-2* mRNA the oligonucleotide may have the sequence

5'-CCCAGCGTGCGCCATCCTTCCC-3' (SEQ3)

It is further preferred if the means for preventing or reducing the
25 expression of the said anti-oncogene or proto-oncogene in the said tumour cell is provided by a DNA construct which expresses an antisense RNA.

Thus, an alternative strategy to blocking expression of the said anti-oncogene or proto-oncogene using antisense oligonucleotides is to
30 introduce into cells vectors that drive expression of antisense RNAs to the

said oncogenes. A suitable RNA to express would be the antisense sequence complementary to the entire oncogene open reading frame. This may be inserted into an appropriate promoter-driven vector, for example a CMV promoter-driven vector, and appropriate mRNA cap and poly A
5 recognition sequences added to the antisense construct at 5' and 3' positions respectively (Kaufman, 1990b). The construct may be delivered to tumour cells using an amphotropic retrovirus vector based system.

It is preferred if the said anti-oncogene or proto-oncogene is *bcl-2* and the
10 said antisense RNA is expressed from the sequence complementary to the entire *bcl-2* open reading frame.

In still further preference, a means for competing with the said inhibitor for the site of action of the said inhibitor may be provided by a DNA
15 construct expressing a mutant of the said inhibitor which interacts with the normal site of action but in a futile and ineffective way, or by introducing directly into the cell a mutant of the said inhibitor. For example, "dominant negative" mutants of Bcl-2 may be useful in the practice of the invention. By site-directed mutagenesis of the Bcl-2 protein inactive
20 mutants of Bcl-2 may be identified which nonetheless interact with normal targets for the Bcl-2 protein but in a futile and ineffective way. These would then constitute "dominant negative" Bcl-2 mutants because if expressed in cells they may compete with normal active Bcl-2 for essential cellular targets of Bcl-2 action and hence block its function. Introduction
25 of such mutants into cells constitute an alternative strategy for interfering with Bcl-2 function.

DNA constructs expressing either antisense RNA or mutants of the inhibitor as described above may be introduced into the target cells in
30 known ways.

For example, the constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the tumour cell (see, for example, Kuriyama *et al* (1991) *Cell Struc. and*
5 *Func.* 16, 503-510, in which purified retroviruses are administered, and Culver *et al* (1992) *Science* 256, 1550-1552, in which cells which produce retroviruses are injected into the tumour) or methods involving simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time.
10 Examples of the latter approach include (preferably tumour-cell-targeted) liposomes (see Nässander *et al* (1992) *Cancer Res.* 52, 646-653) and adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *et al*, *Human Gene Therapy*, April 1992). It may be desirable to locally perfuse a tumour with the delivery vehicle (for example the
15 retrovirus) for a period of time.

It is envisaged that the methods of the invention will find use in the area of human medicine, but may be used to treat other mammals such as horses, cattle, pigs, sheep, dogs, cats, and other vertebrates such as
20 chickens.

Although the survival-promoting functions of *bcl-2* are well described in lymphoid cells, together with the implication that abrogation of *bcl-2* function will re-introduce susceptibility to apoptosis, there has been no
25 suggestion that this may be a generally applicable situation with respect to multiple tissue types. Moreover, the combination of *bcl-2* abrogation together with chemo- or radiotherapy is an important feature of the invention as herein disclosed in Example 3.

30 Inhibition of *bcl-2* expression during therapy may be imposed repeatedly,

for example prior to adjuvant drug therapy. It may be used to sensitize tumour cells to the cytotoxic effects of chemo- and radiotherapy and can therefore be administered (either systemically or more specifically - for example through certain major blood vessels or introduced into specific
5 body cavities) shortly (a few hours) before drug/radiation treatment. The half-life of the Bcl-2 protein is quite long, so the antisense inhibition should preferably be imposed for several hours (for example 6-12 hrs).

Thus, in a further embodiment the tumour cells treated by the methods of
10 the invention are additionally exposed to cytostatic or cytotoxic agents.

Suitable agents are radiation, 6-mercaptopurine, vincristine, vinblastine, etoposide, carboplatin, doxorubicin, cisplatin, fluoruracil, methotrexate, epirubicin, prednimustine, estramustine, bleomycin, mitoxantrone,
15 mitomycin, doxifluridine, carboplatin, bisantrene, pirarubicin, ICRF-187, trimetrexate, idarubicin, bestabucil or KS1/4 DAVLB. These may be used before, during or, preferably, following treatment using the methods of the invention.

20 A second aspect of the invention provides a composition comprising an antisense oligonucleotide adapted to bind to an anti-oncogene or proto-oncogene or transcription product thereof and inhibit transcription or translation, and means to introduce the oligonucleotide into a mammalian tumour cell.

25

A third aspect of the invention provides a composition comprising a DNA construct capable of expressing antisense RNA adapted to bind to an anti-oncogene or proto-oncogene or transcription product thereof and inhibit transcription or translation thereof, and means to introduce the DNA
30 construct into a mammalian tumour cell.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

The composition of the invention (that is to say including the antisense oligonucleotides, DNA constructs and so on) may be administered in any suitable way, usually parenterally, for example intravenously, intraperitoneally or, preferably (for bladder cancers), intra-vesically (ie into the bladder), in standard sterile, non-pyrogenic formulations of diluents and carriers, for example isotonic saline (when administered intravenously). If needed, because the compound of the invention may be immunogenic, cyclosporin or some other immunosuppressant can be administered to provide a longer period for treatment but usually this will not be necessary.

As disclosed above, and also described in the Examples, *bcl-2* may exert its survival promoting effects even in cell types in which it is not normally expressed. By analogy, therefore, expression of *bcl-2* may also serve to render cells of many different lineages more resistant to death by overgrowth, cytokine abuse or other stress when in culture.

A fourth aspect of the invention comprises a cell which is a cell line or a

parent for a cell line, the cell comprising means for expressing the function of the *bcl-2* gene.

By "means for expressing the function of the *bcl-2* gene" we mean means
5 for blocking programmed cell death, especially *myc*-induced cell death and particularly *c-myc*-induced cell death. Such a means includes a construct which expresses a polypeptide with substantially the same anti-apoptotic properties of the *bcl-2* polypeptide. Such polypeptides include fragment
10 or homologues of the *bcl-2* polypeptide which retain the anti-apoptotic properties of the *bcl-2* polypeptide, and include the genes of certain viruses whose function appears to be to block apoptosis of host cells, such as adenovirus p19E1B (Rao *et al*, 1992); Epstein Barr Virus LMP-1 (Gregory *et al*, 1991); Epstein Barr Virus BHRF1 (Pearson *et al*, 1987); herpes simplex virus ICP34.5 (Chou & Roizman, 1992); and baculovirus
15 p35 (Clem *et al*, 1991). Other *bcl-2*-like anti-apoptotic genes may be identified using the assay system disclosed below.

Suitably the cell is derived from a multicellular organism, including mammals such as man, monkey, mouse, rat, hamster or from insects.
20 Mammalian cells are preferred.

Typically, the DNA encoding Bcl-2 may be joined to a wide variety of other DNA sequences for introduction into an appropriate cell line. The companion DNA will depend upon the nature of the host, the manner of
25 the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into a vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the
30 DNA may be linked to the appropriate transcriptional and translational

regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the host cells will be transformed by the vector.

5 Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector,

10 which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings

15 disclosed herein to permit the expression of Bcl-2.

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to

20 be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an

25 alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with

30 their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

10

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

15 A desirable way to modify the DNA encoding Bcl-2, and to facilitate its subcloning, is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491.

20 In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

25 The entire coding region of the human *bcl-2* gene *bcl-2a* is cloned into a suitable expression vector that will direct constitutive and high level expression from one of a range of transcriptional promoters. A drug resistance marker is included in the *bcl-2* construct to allow selection for transfected or infected DNA. Typical promoters that may be used to drive
30 Bcl-2 expression in cells include: (1) the Moloney Murine leukaemia

virus (MoMuLV) LTR which is active in many mouse and human epithelial, mesenchymal and haematopoietic cells (Morgenstern and Land, 1991); (2) the cytomegalovirus (CMV) promoter which is extremely promiscuous and powerful (Boshart *et al*, 1985); (3) a range of cell lineage-specific promoters and enhancer elements may be used to direct tissue-specific expression of *bcl-2*. For example: (i) Myocyte-specific (Abulafia *et al*, 1984); (ii) Pituitary-specific (Schoderbek *et al*, 1992); (iii) Skeletal muscle-specific (Muscat *et al*, 1992; Prody and Merlie, 1991); (iv) Melanocyte-specific (Aguilera *et al*, 1987); (v) Erythroid-specific (Alitalo, 1985); (vi) Eye lens-specific (Alitalo *et al*, 1983); (vii) Fat cell-specific; Akey and Goldfarb, 1989); (viii) Liver-specific (Akey, 1989); (ix) Neuronal-specific (Scholnick *et al*, 1991).

Typical drug resistance markers are the genes encoding resistance to the antibiotics neomycin (G418), puromycin or hygromycin (Kaufman, 1990a). The Bcl-2 constructs are introduced into recipient cells either by transfection using calcium phosphate precipitation (Sambrook *et al*, 1989), lipofection (Felgner *et al*, 1987) or electroporation (Bertling *et al*, 1987) and stably expressing clones isolated under drug selection. If the Bcl-2 constructs are retroviral vectors, the vector DNA is introduced into an appropriate ecotropic (for rodent cells) or amphotropic (for human cells) packaging line and stably transfected packaging cells isolated under drug selection. Virus are then isolated from culture supernatants of the transfected packaging cells and used to infect recipient cells using standard procedures (Morgenstern & Land, 1991). Cells containing stably integrated and expressed retrovirally introduced *bcl-2* are isolated under drug selection (Kaufman, 1990a).

Reagents useful in transfecting vertebrate cells include calcium phosphate and DEAE-dextran or liposome formulation, available from Stratagene

Cloning Systems or Life Technologies Inc, Gaithersburg, MD 20877, USA.

5 Nutrient media useful for culturing transformed host cells are well known in the art and can be obtained from several commercial sources.

Clones stably expressing Bcl-2 protein are isolated and assayed for Bcl-2 expression by immunocytochemical and immunoblotting techniques (Evan & Hancock, 1985; Evan *et al*, 1984) using Bcl-2-specific antibodies raised
10 against synthetic peptides containing Bcl-2 residues 41-55 or 66-78 (see Example 3).

It is preferred if the means for expressing the *bcl-2* encoded polypeptide is integrated into the cell's chromosome or the cell's mitochondrial
15 genome.

Although the use of retroviral vectors is suitable for carrying out the invention, their use may not satisfy the appropriate regulatory authorities.

20 It is preferred if the means for expressing the *bcl-2* encoded polypeptide does not comprise a retrovirus.

Many cells used in culture are immortalized cells or cells derived from tumours, for example myeloma cells which are used to create hybridomas.
25 In a preferred embodiment of the invention the DNA construct expressing Bcl-2 are introduced into the immortalized cell and that transfected cell is then cloned. Thus, the daughter cells, as well as the parent cell, form part of the invention.

30 Cells in which the invention may be practised include all cells from

multicellular organisms which are cultured, particularly those which require careful control of complex growth media, and in which the *myc* gene (which may be *c-myc*, *L-myc*, or *N-myc* but is preferably *c-myc*) is deregulated and overexpressed. These include human (eg HeLa) simian
5 (eg Vero), canine (eg MDCK), rodent (eg Chinese Hamster Ovary) and insect cells.

Insect cells are now commonly used to produce heterologous proteins that are expressed using baculovirus vectors and the like. The present
10 invention may be used to enhance the survival in culture of insect cells such as the Sf9 cell.

The use of insect cells to express gene products is reviewed in Fraser
(1992) *Curr. Top. Microbiol. Immunol.* 158, 131-172 and Murhammer
15 (1991) *Appl. Biochem. Biotechnol.* 31, 283-310.

It is likely that cell death pathways are conserved throughout evolution, and that *bcl-2* may be involved in apoptosis in plants. Accordingly, it is believed that expression of anti-oncogenes or proto-oncogenes, such as
20 *bcl-2*, in plant cells in culture may be beneficial and enhance their survival. Thus, in one embodiment plant cells may be transfected with a Bcl-2 expressing construct and then cultured. Plant cells are useful for expressing protein products in culture and are also useful for the production of secondary metabolites. Methods of culturing plant cells and
25 producing secondary metabolites from such cells are known in the art.

The invention may be beneficial in extending the life of those cell lines in culture that express useful and valuable products. It will be appreciated that more and more pharmaceutical products (for example, tissue
30 plasminogen activator (tPA), erythropoietin (EPO), interleukin, interferon,

tumour necrosis factor), enzymes and the like are polypeptides expressed in cell culture; in particular many polypeptide products may only be expressed effectively from cell lines derived from higher eukaryotes, such as mammals. This may be due to necessary post-translational
5 modifications, such as glycosylation, that occur.

Thus, a fifth aspect of the invention provides a method of producing a product from a cultured cell line, the cells comprising means for expressing the function of the *bcl-2* gene.

10

The methods and cell lines of the invention therefore provide a means of extending the culture life of the cell line expressing the valuable product, and may reduce the cost of propagating such cells by reducing the requirements for expensive culture medium including cytokines,
15 hormones, growth factors and the like.

It will be further appreciated by those skilled in the art that existing cell lines which express a polypeptide product may benefit from the introduction of *bcl-2*. Alternatively, a cell line may be generated that
20 expresses *bcl-2* and has an enhanced life in culture, and that cell line may then be transfected with DNA constructs expressing the desired polypeptide product.

The invention will be particularly beneficial in the production of
25 monoclonal antibodies. Monoclonal antibodies are produced from hybridoma cells (as disclosed herein in respect of the generation of binding moieties to target cells). Thus, introduction of *bcl-2* into existing hybridomas may enhance their life in culture and lead to more effective production of the monoclonal antibody. Alternatively, the *bcl-2* gene may
30 be introduced into a parent myeloma cell line, and this myeloma cell line

used in fusions to make the specific hybridoma expressing the desired monoclonal antibody.

The *bcl-2* gene may be introduced into suitable myeloma parent cells such as NS0, NS-1 and SP2/0, which do not express *bcl-2* mRNA or Bcl-2 protein.

The *bcl-2* gene may be introduced into suitable hybridoma cells expressing antibodies useful in diagnostics (for example in blood-typing, pregnancy testing, immunocytochemistry, immunoscintigraphy); those expressing antibodies used in preparative procedures (eg immunopurification, quality control); those used therapeutically and those used in research. The benefits of expressing Bcl-2 in existing hybridomas may be to produce higher yields of antibody, exhibit less capriciousness in the growth of the secreting hybridoma in culture, show greater resistance to transient or long term adverse culture conditions, grow to and survive at higher densities *in vitro*, show more consistent growth in culture over time and from occasion to occasion, and have decreased requirements for foetal calf serum and expensive cytokine additives.

Examples of hybridoma cells expressing monoclonal antibodies, which may be usefully transfected with the *bcl-2* gene are shown in Table 2. They are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852-1776, USA.

Table 2

	Monoclonal antibody reactive to:	Ig Class	Designation	ATCC No
5	Human acetylcholinesterase	IgG ₁	mAb 35	TIB 175
	Colorectal carcinoma	IgG ₁	1116-NS-19-9	HB 8059
	Insulin	IgG ₁ κ	CC9C10	HB 123
	Interleukin-2	IgM κ (rat)	7D4	CRL 1698
10	Schistosoma mansoni glycoprotein	IgG ₁	130C3/2B/8	HB 8088
	Transferrin receptor	IgG _{2a}	L5.1	HB 84
	Hepatitis B, surface antigen	IgG ₁	H2SB10	CRL 8017

15

Growth conditions for cells in culture are well known in the art. A typical standard growth medium for mammalian cells is the Dulbecco's modified Eagle medium (Dulbecco & Freeman (1959) *Virology* 8, 396):

20	<u>Component</u>	<u>mg/L</u>	<u>Component</u>	<u>mg/L</u>
	Inorganic Salts:		Amino Acids:	
	CaCl ₂ .2H ₂ O	265.00	L-Arginine.HCl	84.00
	Fe(NO ₃).9H ₂ O	0.10	L-Cystine	48.00
25	KCl	400.00	L-Glutamine	584.00
	MgSO ₄ .7H ₂ O	200.00	Glycine	30.00
	NaCl	6400.00	L-Histidine HCl.H ₂ O	42.00
	NaHCO ₃	3700.00	L-Isoleucine	105.00
	NaH ₂ PO ₄ .2H ₂ O	141.00	L-Leucine	105.00
30	Other Components:		L-Lysine HCl	146.00
	D-Glucose	4500.00	L-Methionine	30.00

29

	Phenol Red	15.00	L-Phenylalanine	66.00
	Aminopterin	0.176	L-Serine	42.00
	Hypoxanthine	13.60	L-Threonine	95.00
	Thymidine	3.88	L-Tryptophan	16.00
5			L-Tyrosine	72.00
			L-Valine	94.00
			Vitamins:	
			D-Ca panthothenate	4.00
			Choline Chloride	4.00
10			Folic Acid	4.00
			i-Inositol	7.20
			Nicotinamide	4.00
			Pyridoxal HCl	4.00
			Riboflavin	0.40
15			Thiamine HCl	4.00

RPMI 1640 medium is also suitable for mammalian cells (Moore *et al* (1967) *A.M.A.* 199, 519).

20	<u>Component</u>	<u>mg/L</u>	<u>Component</u>	<u>mg/L</u>
	Inorganic Salts:		Amino Acids:	
	Ca(NO ₃) ₂ ·4H ₂ O	100.00	L-Arginine HCl	200.00
	KCl	400.00	L-Asparagine	50.00
	MgSO ₄ ·7H ₂ O	100.00	L-Aspartic Acid	20.00
25	NaCl	6000.00	L-Cystine	50.00
	NaHCO ₃	2000.00	L-Glutamic Acid	20.00
	Na ₂ HPO ₄	800.00	L-Glutamine	300.00
	(anhyd.)		Glycine	10.00
	Other Components:		L-Histidine	15.00
30	D-Glucose	2000.00	(free base)	

30

	Glutathione	1.00	L-Hydroxyproline	20.00
	(reduced)		L-Isoleucine	50.00
	Phenol Red	5.00	(allo free)	
5			L-Leucine	50.00
			(methionine free)	
			L-Leucine.HCl	40.00
			L-Methionine	15.00
			L-Phenylalanine	15.00
			L-Proline (hydroxy	20.00
10			L-proline free)	
			L-Serine	30.00
			L-Threonine	20.00
			(allo free)	
			L-Tryptophan	5.00
			L-Tyrosine	20.00
15			L-Valine	20.00
	Vitamins:			
			Biotin	0.20
			D-Ca Pantothenate	0.30
20			Choline Chloride	3.00
			Folic Acid	1.00
			i-Inositol	35.00
			Nicotinamide	1.00
			Para-aminobenzoic	1.00
			Acid	
25			Pyridoxal HCl	1.00
			Riboflavin	0.20
			Thiamine HCl	1.00
30			Vitamin B ₁₂	0.005

RPMI 1640 may be supplemented, for example with 10% foetal calf serum, or with other growth factors as necessary. In the production and maintenance of hybridoma cells a HAT supplement is usually added to a concentration of hypoxanthine 0.1 mM; aminopterin 0.4 μ M; and thymidine 16 μ M. After fusion, cells are immediately cultured in HAT medium for 1 to 2 weeks.

A typical growth medium for insect cells is Grace's medium (Grace (1962) *Nature* 195, 788):

Component		mg/L		Component		mg/L	
Inorganic Salts:				Vitamins:			
	CaCl ₂ .2H ₂ O	993.00		Para-aminobenzoic acid		0.02	
	KCl	4100.00					
15	MgCl ₂ .6H ₂ O	2280.00		Biotin		0.01	
	MgSO ₄ .7H ₂ O	2780.00		D-Calcium pantothenate		0.02	
	NaHCO ₃	350.00		Choline Chloride		0.20	
	NaH ₂ PO ₄ .2H ₂ O	1150.00		Folic Acid		0.02	
Other Components:				i-Inositol		0.02	
20	Fructose	400.00		Niacin		0.02	
	Fumaric Acid	55.00		Nicotinic Acid		0.02	
	D-Glucose	700.00		Pyridoxine, HCl		0.02	
	α -Ketoglutaric Acid	370.00		Riboflavin		0.02	
25	Malic Acid	670.00		Thiamine, HCl		0.02	
	Succinic Acid	60.00					
	Sucrose	26680.00					
Amino Acids:							
	L-Alanine	225.00					
30	β -Alanine	200.00					

	L-Arginine.HCl	700.00
	L-Asparagine	350.00
	L-Aspartic Acid	350.00
	L-Cystine	22.00
5	L-Glutamic Acid	600.00
	L-Glutamine	600.00
	Glycine	650.00
	L-Histidine	3335.00
10	HCl.H ₂ O	
	L-Isoleucine	50.00
	L-Leucine	75.00
	L-Lysine.HCl	625.00
	L-Methionine	50.00
15	L-Phenylalanine	150.00
	L-Proline	350.00
	DL-Serine	1100.00
	L-Threonine	175.00
	L-Tryptophan	100.00
20	L-Tyrosine	50.00
	L-Valine	100.00

A sixth aspect of the invention provides a product prepared by a method according to the fifth aspect of the invention.

25

Mammalian and insect cells have been used to express many valuable polypeptide products. For example, recombinant human nerve growth factor (NGF) is obtained by expression of the human NGF gene in mammalian as taught in US 5 082 774; recombinant interleukin-6 (IL-6)

30 is expressed by eukaryotic host cells to have the same glycosylation and

phosphorylation as native IL-6 as taught in US 7 612 675; human platelet-derived growth factor (PDGF) can be expressed in mouse fibroblast cells as taught in US 7 218 276; CD4 (T cell surface protein T4) may be expressed in Lepidoptera insect cells as taught in US 5 155 037; and
5 human interferon-gamma may be expressed in mammalian cells as taught in US 4 939 088 or in insect cells as taught in US 5 147 788. We believe that all of these cell lines, and others expressing recombinant polypeptides, may benefit in culture from the introduction of a DNA construct expressing Bcl-2. These patents and all other references are incorporated
10 herein by reference.

Compounds that modulate apoptosis are of interest. Those compounds that are agonists (inducers) may allow for controlled death of cells either in culture, or in a patient who will benefit from such controlled cell death,
15 for example, death of tumour cells. Those compounds that are antagonists (blockers) may prevent death of cells in culture or in a patient who will benefit from the prevention of cell death, for example, in a patient with a degenerative disease.

- 20 Screening for antagonists of apoptosis requires a reproducible and consistent system for inducing apoptosis in target cells. Compounds that block apoptosis will yield surviving cells whereas cells not treated with the compound will substantially all die.
- 25 Requirements for an assay system for anti-apoptotic compounds can be summarized: (1) an indicator cell line that can be induced to undergo apoptosis; (2) a defined and reproducible system for inducing apoptosis, with a very low background of cell death in uninduced cells; and (3) a quantitative assay for cell viability or cell death. A system meeting these
30 requirements is described later.

Thus, an eighth aspect of the invention provides an assay for detecting whether a compound is involved in modulating apoptosis, the assay comprising a cell transformed with a DNA construct comprising a proto-oncogene coding sequence encoding a polypeptide which, when forcibly
5 expressed or activated, induces apoptosis, and regulatory elements which allow transcription of the coding sequence.

By "compound" we include a gene, which may be transfected into the transformed cell of the assay, polypeptides which may act as cell growth
10 modulators such as cytokines, and low molecular weight compounds and small molecules, for example those of $M_r < 1000$.

The forcible expression of the proto-oncogene coding sequence may conveniently be accomplished using an inducible promoter. For example,
15 the well known glucocorticoid/dexamethasone-inducible MMTV or metallothionein promoters inducible by cadmium or zinc may be used to drive *myc* expression. Alternatively, the tetracycline regulatable system of Dingeramn *et al* (1992) *Mol. Cell. Biol.* 12, 4038-4045 or the isopropyl thio- β -D-galactopyranoside (IPTG)-inducible system of Baim *et*
20 *al* (1991) *Proc. Natl. Acad. Sci. USA* 88, 5072-5076 may be used.

Alternatively, and preferably, the proto-oncogene polypeptide may be forcibly activated. For example, fusion of the *myc* protein with fragments of the oestrogen receptor (ER) protein may yield a protein which only
25 produces its *myc* activity when it is activated by an oestrogen such as β -oestradiol.

In a preferred embodiment the proto-oncogene is *myc*; the *myc* gene may be *c-myc*, *L-myc* or *N-myc* but is preferably *c-myc*.

DNA containing the N-*myc* sequence may be obtained using the methods described in Taya *et al* (1986) *EMBO J.* incorporated herein by way of reference and DNA containing the L-*myc* sequence may be obtained using the methods described in De Pinho (1987) *Genes Dev.* 1, 1311-1326.

5

The invention will now be described in detail with reference to the following examples and figures wherein:

Figure 1 shows growth curves of Rat-1 fibroblasts either with or without constitutive *c-myc* expression in various concentrations of FCS.

10

Triplicate cultures of control Rat-1 cells or Rat-1 cells constitutively expressing wild type *c-myc* or *c-myc* mutants were cultured in medium containing the various levels of FCS shown and live cells counted at daily intervals. Mean values of the triplicates, together with standard errors, are shown plotted against time.

15

Figure 2 shows that constitutive *c-myc* expression prevents growth-arrest in serum-deprived Rat-1 cells.

20

Triplicate logarithmic cultures of Rat-1/*neo* control and Rat-1/*myc* cells were transferred into medium containing either 10% or 0.1% FCS. After 48 hours, cells were labelled for 1 hour with 2mM BrdU, trypsinised, fixed in ethanol and stained with propidium iodide and appropriately conjugated anti-BrdU antibody. Flow cytometric analysis was carried out on a Beckton-Dickinson FACSstar plus.

25

- A. DNA histograms of Rat-1 cells.
- B. Percentage of cells staining with anti-BrdU antibody (ie traversing S phase) over a one hour period.

30

Figure 3 shows that Myc induces death by apoptosis in serum-deprived Rat-1 cells.

- Figure 3A: Rat-1/c-myc cells were transferred to medium containing 0.1% FCS and observed by time-lapse cinemicroscopy at a rate of one frame every 30 seconds. Representative frames from a typical apoptotic event are shown with the time in minutes given from the last frame when the cell appeared normal.
- Figure 3B: Cell death is accompanied by nucleosome laddering. Rat-1 cells constitutively expressing either active $\Delta 144-262$ (lanes 2, 4 and 6) or inactive $\Delta 106-144$ (lanes 3, 5 and 7) c-myc mutants were transferred to medium containing 0.1% FCS. Dying cells were harvested at various times after transfer, by virtue of their reduced adherence. In cultures with no dying cells, very few cells were harvested by this method. DNA isolated and fractionated on a 1.5% agarose gel. Lane 1 - standards; Lanes 2 and 3 - 0 hours; Lanes 4 and 5 - 30 hours; Lanes 6 and 7 - 40 hours; Lane 8 - dexamethasone treated thymocytes.
- Figure 3C: Electron microscopic analysis of individual Rat-1/myc cells undergoing apoptosis in low serum. Frame 1 shows a normal viable Rat-1/myc cell. The nucleus is marked N. Frames 2 to 4 are representative EM micrographs of Rat-1/myc cells at progressively more advanced stages of apoptosis and exhibiting cytoplasmic and nuclear vesicularisation.
- Figure 4 shows that apoptosis in serum-deprived Rat-1/myc cells is dependent upon active Myc protein.
- Log-phase Rat-1 cells constitutively expressing either full length c-myc protein or the deletion mutant $\Delta 106-143$ fused to estrogen receptor were

transferred to medium containing 0.1% FCS either with or without 2nm β -oestradiol. After 3 days, cultures were examined by phase microscopy.

Figure 5 shows that deregulated Myc expression induces apoptosis in serum-deprived primary cells.

Rat Embryo Fibroblasts constitutively expressing *c-myc* were cultured in medium containing 0.1% FCS for 72 hours. At various times, the culture was inspected microscopically. A - t = 0 hrs; B - t = 24 hrs; C - t = 48 hrs; D - t = 72 hrs.

Figure 6 shows that the degree of apoptosis in serum-deprived Rat-1/*myc* cells is dependent upon intracellular level of Myc protein.

- Figure 6A: Sparse exponential cultures of each clone growing in 10% FCS were transferred to medium containing various concentrations of FCS and inspected for apoptosis by phase microscopy after 3 days. Results are shown from three independent Rat-1 clones chosen on the basis of level of expression of *c-myc* protein (Penn *et al*, 1990). Clone 21 expresses ~5,000 Myc molecules per cell (mpc), clone 19 ~12,000 mpc, and clone 26 ~18,000 mpc. Four other clones, 2 (4,000 mpc), 5 (5,500 mpc), 12 (7,000 mpc) and 11 (14,000 mpc) were also studied but data for them is not shown.
- Figure 6B: The rate of apoptotic events in each of the three Rat-1/*myc* clones is shown after transfer to medium containing 0.1% FCS. 75 randomly picked live cells were selected at the start of the experiment and these were followed by time-lapse cinemicroscopy at a rate of 12 frames per hour. At the end of each 2 hour (24 frame) time interval, the total number apoptotic events (top) and the total number of live cells were

summed and plotted against time.

Figure 7 shows Myc-dependent apoptosis in Rat-1 fibroblasts growth arrested by various means.

5

Logarithmically growing subconfluent Rat-1/*myc*-ER and Rat-1/ Δ 106-143 *myc*-ER cells were growth-blocked by the following means, either in the presence of absence of 2mM β -oestradiol as indicated:

- 10 i. Thymidine block: DME containing 10% stripped FCS plus 2mM thymidine
- ii. Isoleucine starvation: isoleucine-free DME containing 10% dialysed and stripped FCS
- 15 iii. Interferon: DME containing 10% stripped FCS plus 2,000 units/ml recombinant rat γ -interferon
- iv. Cycloheximide: DME containing 10% stripped FCS plus 50 μ g/ml cycloheximide

Figure 8 shows that ectopic Myc activation induces apoptosis in Rat-1/*myc*-ER fibroblasts already growth-arrested by serum deprivation, thymidine block or isoleucine starvation.

Sub-confluent Rat-1/*myc*-ER fibroblasts were growth-arrested either by serum deprivation (0.1% FCS for 48 hrs), thymidine block (2mM thymidine for 48 hrs) or isoleucine starvation (60 hrs). Cells were observed by time-lapse cinemicroscopy for the last 40 hours of this starvation period which revealed essentially a complete absence of cell division. Growth arrest was further confirmed by flow cytometry (not shown). The medium was then changed and replaced with the same growth-blocking medium either with or without 2mM β -oestradiol. The

cells were then observed for a subsequent 35 hours and apoptotic cell deaths recorded and summed for each 1 hour period. The cell number indicated at "start" is the actual number of live cells followed from the time of the start of recording.

5

Figure 9 shows the determination of expression of Bcl-2a protein by immunoblotting.

Figure 10 shows the results of time-lapse cinemicroscopic quantitation of apoptosis in Rat-1 cells.

10

Figure 11 shows the time-lapse cinemicroscopic analysis of the effect of Bcl-2 expression on Myc-induced proliferation of Rat-1 cells.

Figure 12 shows that constitutive Bcl-2 expression protects Rat-1/Myc cells from thymidine and VP16/etoposide-induced apoptotic cell death.

15

Figure 13 shows the *bcl-2* open reading frame and translation thereof.

Figure 14 shows the effect of *bcl-2* expression on myeloma parent NS1 survives at high density.

20

Figure 15 shows the survival of myeloma parent NS1 in foetal calf serum (FCS).

25

Example 1: Induction of apoptosis in fibroblasts by *c-myc* protein.

Constitutive c-myc expression prevents growth arrest in serum-deprived Rat-1 fibroblasts and induces cell death.

30

Growth arrest is accompanied by rapid down-regulation of *c-myc* expression. We therefore investigated the ability of Rat-1 cells containing a normal human *c-myc* gene driven by a constitutive promoter, and therefore unable to down-regulate *c-myc* expression, to arrest when
5 deprived of mitogens. Control Rat-1 cells (Rat-1/*neo*) and Rat-1 cells constitutively expressing wild-type *c-myc* (Rat-1/*myc*) or mutant *c-myc* were cultured at various serum concentrations and numbers of live cells counted at daily intervals (Figure 1). The *c-myc* deletion mutant $\Delta 106$ -
143 is inactive in transformation and autosuppression assays whereas
10 $\Delta 145$ -262 is active (Penn *et al.*, 1990; Stone *et al.*, 1987). Growth curves of all the cells appeared very similar, irrespective of whether or not the cells constitutively expressed active *c-myc*. This suggests that constitutive
15 *c-myc* expression has no effect on the ability of Rat-1 cells to slow their growth in low serum levels. To confirm this finding, we examined the growth status of Rat-1 cells and Rat-1/*myc* cells in low serum for DNA
content and bromodeoxyuridine (BrdU) incorporation by flow cytometry. The results of this analysis are shown in Figure 2 and, paradoxically,
appear to show that there is a complete block to growth arrest in cell
20 constitutively expressing *c-myc*. To accomodate these two apparently conflicting results, we examined the possibility that Rat-1/*myc* cells were
dying in low serum. Microscopic inspection of such cultures revealed this to be the case.

Myc-induced cell death occurs by apoptosis.

25

The manner of cell death of Rat-1/*myc* cells in low serum was examined by time-lapse cinemicroscopy. Logarithmic Rat-1/*myc* cells were transferred into medium containing 0.1% FCS and a field of about 120 cells observed. Individual cell deaths occurred apparently randomly
30 throughout the culture. The first cell deaths were observed within 30.

minutes of serum withdrawal and cell deaths then continued at a more or less constant rate, typically ending with death of the entire culture after about 6-9 days. Each death is rapid - the interval between apparent morphological normality and complete cell fragmentation is typically about 5 30 minutes (Figure 3A). Death begins with loss of cell-cell contact. This is followed by nuclear condensation, membrane blebbing, cytoplasmic condensation, and ends in cell fragmentation. All these characteristics were confirmed by transmission electron microscopy of dying cells (Figure 3C). DNA from dying cells was analysed and revealed fragmentation of 10 chromatin into nucleosome ladders (Figure 3B). All the features described above are diagnostic of a form of programmed cell death termed apoptosis (Bursch *et al*, 1990; Wyllie, 1987). Identical apoptosis was seen in Rat-1/*myc* cultures in which serum was replaced with a defined serum substitute (not shown) lacking mitogens and unable to support fibroblast 15 growth (Waters *et al*, 1991). Moreover, Rat-1/*myc* fibroblasts could be viably maintained in serum-free media supplemented only with specific defined growth factors (not shown). Both of these observations argue that death is due to absence of necessary growth factors rather than to depletion of metabolites.

20

Apoptosis is dependent upon active Myc expression.

Thus far, all experiments had been conducted using independently isolated Rat-1 clones. To confirm that cell death was a property of *c-myc* 25 expression rather than clonal variation, we examined cell death in Rat-1 cells constitutively expressing Myc-estrogen receptor chimaeras (Rat-1/*myc*-ER cells). In these cells, activity of the chimaeric Myc protein is completely dependent upon the availability of exogenous β -oestradiol (Eilers *et al*, 1989). In the absence of β -oestradiol, Rat-1/*myc*-ER cells 30 arrest in low serum in a G_0/G_1 -like state (not shown) and remain viable for



several weeks. In the presence of 2nm β -oestradiol, however, the rapid onset of apoptosis is observed in low serum. In contrast, Rat-1 fibroblasts expressing the transformation-defective mutant of Myc Δ 106-143 (Penn *et al*, 1990; Stone *et al*, 1987) fused to ER (Rat-1/ Δ 106-143-ER cells) arrest in low serum and exhibit no apoptosis irrespective of the presence of β -oestradiol (Figure 4). Thus, apoptosis of Rat-1/*myc*-ER depends upon the presence of active Myc protein and is not a trivial result of the addition of β -oestradiol to the culture.

10 *Deregulated expression of Myc induces apoptosis in serum-deprived Rat Embryo Fibroblasts.*

Rat-1 cells are an immortalised and established cell line. We were therefore interested to determine how general was Myc-induced apoptosis; in particular, whether it occurred in a non-established primary fibroblast culture. Accordingly, Primary Rat Embryo Fibroblasts (REFs) constitutively expressing Myc were subjected to serum deprivation and monitored microscopically over a 72 hour period. As with Rat-1/*myc* cells, such REF/*myc* fibroblasts fail to arrest growth in low serum as determined by flow cytometry (not shown). As can be seen in Figure 5 substantial apoptosis occurs within 24 hours of transfer into low serum.

Extent of Myc-induced apoptosis is related to the levels of Myc protein in cells.

25 We next investigated whether there was any correlation between intracellular Myc protein level and susceptibility to Myc-induced apoptosis in Rat-1/*myc* cells. Various Rat-1/*myc* cell clones were selected, each of which expresses a different steady-state level of Myc protein (Penn *et al*, 1990). Each clone was then assayed for apoptosis by two independent

30

assays. First, cells were cultured in various concentrations of FCS and the degree of cell death was assessed by microscopic examination after 3 days. Results are shown in Figure 6A for the three Rat-1/*myc* clones 21, 19 and 26, which representatively span the range of Myc protein levels investigated. Clone 26 expresses most Myc and exhibits significant apoptosis even in serum levels as high as 2%. Apoptosis is even more evident at lower serum levels. In contrast, clone 21, which expresses a level of Myc protein similar to that found in normal fibroblasts (Waters *et al*, 1991), exhibits apoptosis only at the lowest serum levels. Clone 19, with an intermediate Myc protein level, exhibits an intermediate phenotype. Results obtained for 4 other clones (2, 5, 12 and 11), each expressing a different Myc level, were consistent with this trend (not shown). Second, the various Rat-1/*myc* clones were transferred into 0.1 % FCS and fields containing identical numbers of cells monitored for apoptosis by time-lapse cinemicroscopy. This type of analysis is complicated by the fact that cell number is continuously varying because of both cell division and cell death. For this reason, results were pooled within each 2 hour time interval and both cumulative cell deaths and live cell number at the end of each 2 hour interval were plotted against time. Data obtained using clones 21, 19 and 26 is shown in (Figure 6B). The rate of apoptosis is highest in clone 26, intermediate in clone 19, and lowest in clone 21. Again, results obtained with the 4 other tested clones confirmed this positive correlation between higher Myc levels and higher apoptotic rate (not shown).

Thus, we conclude that the sensitivity of Rat-1/*myc* cells to induction of apoptosis upon serum depletion and its rate both depend upon the level of Myc protein expressed. Even the low levels of Myc protein observed in normal Rat-1 fibroblasts are, however, sufficient to induce apoptosis in serum-deprived cells if Myc expression is deregulated.

Regions of the Myc protein required for apoptosis.

Certain regions of the Myc protein are absolutely required for its known activities in co-transformation, autosuppression and inhibition of differentiation (Freytag *et al*, 1990; Penn *et al*, 1990; Stone *et al*, 1987). These regions include the basic-Helix-Loop-Helix-Leucine zipper at the C-terminus and part of the N-terminal region. We examined the ability of a range of Myc mutants to induce apoptosis when expressed constitutively in serum-deprived Rat-1 cells. The results demonstrate a complete concordance between those regions required for apoptosis and those necessary for co-transformation, autosuppression and inhibition of differentiation (Table 3). Thus, the ability of Myc to induce apoptosis is mediated by similar domains of the protein to those involved in other known functions attributed to the Myc protein.

Table 3. Co-transforming and apoptosis inducing activities of Myc mutants.

MYC Mutant	Nature of Mutation	Active in Co-transformation	Active in Inducing Apoptosis
wild type	-	+	+
5 Δ 7-91	Transcriptional modulation?	-	-
Δ 41-53	Loss of 1st Myc homology box	+	+
Δ 106-143	Transcriptional modulation?	-	-
Δ 145-262	No observed effect	+	+
Δ 265-317	No observed effect	+	+
10 Δ 371-412	Deletion of part of helix-loop-helix	-	-
Δ 414-433	Deletion of leucine zipper	-	-
In414	Interruption of leucine zipper	-	-

15 *Constitutive Myc expression induces apoptosis in Rat-1 cells arrested by various means at various points in the cell cycle.*

20 Fibroblast proliferation can be temporarily blocked in a number of mechanistically different ways whilst maintaining viability (reviewed in Pardee, 1989). These include G₀-arrest by serum deprivation, S phase block by thymidine excess, late G₁-block by isoleucine deprivation, interferon arrest in G₁, and transient treatment with cycloheximide (Zetterberg and Larsson, 1985). We examined whether any of these procedures induced apoptosis in Rat-1 cells in a Myc-dependent fashion. Rat-1/myc-ER fibroblasts were maintained in asynchronous subconfluent

logarithmic cultures for several days and then subjected to various types of proliferation block either in the absence or presence of β -oestradiol. Cultures were then examined for apoptosis at various appropriate time points (Figure 7). Application of any of these growth-blocking regimes
5 activated apoptosis in a Myc-dependent manner, although the onset of apoptosis varied depending upon the specific treatment. Significant apoptosis was visible within only four hours of treatment with cycloheximide in cells containing active Myc. In contrast, appreciable
10 apoptosis was visible only after 24-48 hours in cells starved of serum or isoleucine or blocked with thymidine.

We next investigated whether Myc activation would induce apoptosis in cells already arrested and, if so, how rapidly. Rat-1/*myc*-ER cells were
15 growth arrested by serum-deprivation (G_0), isoleucine starvation (G_1) or thymidine-block (S) for 48 hours in the absence of β -oestradiol. Growth arrest and cell cycle position was confirmed by flow cytometric analysis and by BrdU incorporation (not shown). β -oestradiol was then added and the cultures monitored for apoptosis by time-lapse cinemicroscopy (Figure
20 8). Apoptosis is evident within 60 minutes of Myc activation in serum-starved cells and within 3-4 hours of Myc activation in isoleucine-starved or thymidine-blocked cells.

We conclude that Myc activation combined with any tested growth-arrest
25 procedure, either before or after the late G_1 commitment point, leads to the rapid onset of apoptosis. Moreover, it is not necessary for cells to be actively cycling for Myc to activate programmed cell death.

DISCUSSION

Deregulated Myc expression induces apoptosis in fibroblasts blocked in proliferation.

5

Our results demonstrate that c-myc can be a potent inducer of apoptosis in immortalised Rat-1 fibroblasts and primary Rat Embryo Fibroblasts when combined with a block to proliferation. We also see similar behaviour in analogous experiments with Swiss 3T3, NIH 3T3 and mouse embryo
10 fibroblasts (unpublished data of GIE, MB and TDL). Somewhat similar observations have been recently made in a bone marrow derived cell line (Askew *et al*, 1991).

Myc expression induces apoptosis both in proliferating cells upon which
15 a proliferation block is imposed and in cells already arrested and in which Myc is subsequently activated. In both cases, the effect is observed irrespective of the method used to implement growth arrest, whether it be by growth factor or metabolite depletion or by the action of a drug or chalone. Moreover, the fact that rapid initiation of apoptosis by Myc
20 occurs in cells arrested in either G₁ or S phase argues that cells can enter a programmed cell death pathway both before and after the commitment point in late G₁ (Pardee, 1989).

Whenever we observe Myc-dependent apoptosis, cell deaths proceed over
25 a fairly extended time span. This may suggest that, although promoted by Myc, the exact moment of commitment to apoptosis of any individual cell is partially determined by certain stochastic factors. In the case of serum-starved Rat-1/myc cells, those cells not dying continue to proceed through the cell cycle, consistent with established mitogenic properties of c-myc
30 (Eilers *et al*, 1991). In proliferating asynchronous Rat-1/myc cells, the

time of onset of apoptosis varies depending upon the nature of the proliferation block imposed. We presume that this reflects the different rapidities with which various proliferation blocks exert their effect, a presumption consistent with flow cytometric analyses. For example, isoleucine deprivation arrests cells only after about 48 hours, the time we presume it takes to exhaust endogenous isoleucine stores. Apoptosis also becomes evident around this time. On the other hand, serum deprivation tends to arrest fibroblasts when they next enter G₁. As Rat-1 cells have a cell-cycle time of about 15 hours (unpublished observations of GIE and TDL), we expect virtually all cells in an asynchronous culture would pass through G₁ and encounter a signal to arrest within that time, although some would do so much sooner. Consistent with this, apoptosis is first detectable within an hour of serum withdrawal in asynchronous Rat-1/myc cultures after which it continues at a more or less uniform rate. A similarly rapid onset of apoptosis is seen in asynchronous cultures of Rat-1/myc cells in which DNA blocked in S-phase with thymidine. Thus, the combination of constitutive Myc expression and any tested block to proliferation appears to be lethal.

Example 2: Materials and methods relating to Example 1.

Cell Culture and Cell Lines.

The preparation of recominant retroviruses directing constitutive expression of *c-myc*, various *c-myc* mutants and *myc-ER* chimaeras has already been described, as has the isolation of appropriately infected Rat-1 cells (Eilers *et al*, 1989; Eilers *et al*, 1991; Penn *et al*, 1990a,b). Cells were assayed for constitutive expression of both *c-myc* mRNA by RNase protection and Northern blot analysis (Penn *et al*, 1990a,b) and for *c-myc* protein by ELISA and semi-quantitative immunofluorescent confocal

microscopy (Waters *et al*, 1991). All cells were maintained in Dulbecco's modified E4 medium supplemented with 10% foetal calf serum and 1mg/ml Geneticin. Cells were passaged by standard trypsinisation and seeded directly onto tissue culture plastic. Ecotropic viruses directing
5 expression of chimaeras between Myc and truncated estrogen receptor were a kind gift from Drs Martin Eilers and Professor J. Michael Bishop (UCSF, California, USA). Rat-1 cells were infected with retroviruses encoding Myc-estrogen receptor chimaeras and Rat-1 lines expressing wt Myc-ER and $\Delta 106-143$ Myc-ER isolated as described for Rat-1/*myc* lines
10 (Penn *et al*, 1990a,b). Myc-ER and $\Delta 106-143$ Myc-ER clones were maintained in phenol red-free Dulbecco's E4 medium supplemented with 10% charcoal-dextran stripped FCS and 1mg/ml Geneticin. Myc was functionally activated by the addition of β -oestradiol to the medium at a final concentration of $2\mu\text{M}$.

15

Biochemical and analytical techniques.

To examine nucleosome laddering, equal numbers of cells were established in 25 cm^3 tissue culture flasks. Medium was then changed in
20 each flask, as indicated, and at various time points, dying cells were harvested by virtue of their reduced adherence. As a consequence, very few cells were obtained from non-dying cultures, with consequently little DNA. The experiment was thus normalised according to starting cell number rather than to amount of DNA extracted. We chose this method
25 of normalisation because the proportion of ladderred chromatin at any one time is quite small relative to intact chromatin present in those non-apoptotic cells in the culture and this large excess of intact DNA obscures any ladders present. The assay therefore shows when chromatin laddering occurs but the results are in no way quantitative. Instead, quantitation of
30 apoptosis was carried out by time-lapse cinemicroscopy (see below).

DNA extraction and fractionation on 1.5% agarose gels was performed as described (Smith *et al*, 1989).

Standard electron microscopic procedures were used.

5

Time-lapse cinemicroscopy was conducted using a Olympus inverted phase contrast microscopes and images were collected on 16mm monochrome cine film with a cine camera regulated by an external timer. Cell division events were scored at the time at which septa formed between two daughter cells. Apoptotic cell death events were scored midway between the last appearance of normality and the point at which the cell became fully detached and rounded. This corresponds to about $t + 15$ minutes in Figure 3A.

15 **Example 3: Interaction between *c-myc* and *bcl-2* proto-oncogenes.**

The *bcl-2* proto-oncogene is activated by translocation in a variety of B-lymphoid tumours and synergises with the *c-myc* oncogene in tumour progression (Strasser *et al*, 1990). The mechanism of synergy is unclear but Bcl-2 expression has been shown to inhibit apoptosis (Hockenbery *et al*, 1990; Korsmeyer *et al*, 1990) a property presumably pertinent to its proto-oncogenic *modus operandi* (Korsmeyer *et al*, 1990). We have demonstrated in Examples 1 and 2 that the *c-myc* gene is a potent inducer of apoptosis, in addition to its established role in mitogenesis. In this Example we show that expression of the *bcl-2* protein (Bcl-2) specifically abrogates *c-myc*-induced apoptosis without affecting the *c-myc* mitogenic function. This provides a novel mechanism for oncogene cooperation of potential importance both in carcinogenesis and in the evolution of drug resistance in tumours.

30

- Rat-1 fibroblasts constitutively expressing a chimaeric protein comprising a full length cMyc polypeptide fused to part of the human oestrogen-receptor (Rat-1/Myc-ER cells) show demonstrable cMyc activity only in the presence of β -oestradiol (see Examples 1 and 2; Eilers *et al*, 1989; 5 1991). Thus, in the absence of β -oestradiol, serum-deprived Rat-1/Myc-ER cells stably arrest in a G_0 -like state, whereas addition of β -oestradiol prevents arrest and induces rapid apoptotic death. This apoptosis is absolutely dependent upon cMyc expression, its extent is proportional to the level of intracellular cMyc protein, and it requires the same regions of 10 the cMyc protein as are required for co-transformation and autosuppression (see Examples 1 and 2). The role of cMyc in apoptosis is not confined to fibroblasts and has recently also been described in haematopoietic cells (Askew *et al*, 1991).
- 15 To investigate the effect of *bcl-2* expression on cMyc-induced apoptosis we infected Rat-1/cMyc-ER cells with a retrovirus directing constitutive expression of human *bcl-2a* together with a selectable puromycin resistant marker. Puromycin-resistant cells positive for Bcl-2 protein expression (Rat-1/cMyc-ER/Bcl-2 cells) were identified by immunoblotting (Figure 20 9A) and immunocytochemistry (data not shown) using anti-Bcl-2 monoclonal antibodies (Hockenbery *et al*, 1990; Pezella *et al*, 1990). Proliferating Rat-1/cMyc-ER/Bcl-2 fibroblasts and appropriate control cells were then assayed for cMyc-induced apoptosis by microscopic inspection 48 hours after serum depletion. Constitutive Bcl-2 expression 25 effectively abolishes cMyc-induced apoptosis under these conditions (Figure 9B). Essentially identical results were observed with ten individual clones and one clone, clone 5, is used as the example in this study. To quantitate more accurately the inhibition of cMyc-induced apoptosis by Bcl-2, we pre-arrested Rat-1/cMyc-ER and Rat-1/cMyc-ER/Bcl-2 cells in G_0 by serum depletion and then activated cMyc by 30

addition of β -oestradiol. This induces the rapid onset of apoptosis which can be quantitated by time-lapse cinemicroscopy (see Examples 1 and 2). Time-lapse cinemicroscopy also permits confirmation that each death is apoptotic: apoptosis is characterised by its extreme rapidity, typically taking about 30-60 minutes to proceed through a diagnostic sequence of cytoplasmic blebbing, vesicularisation and nuclear condensation (Wyllie, 1987; Bursch *et al*, 1990). Figure 10 demonstrates that Bcl-2 expression completely inhibits the onset of apoptosis in serum-deprived Rat-1 cells following cMyc activation.

If Bcl-2 blocks cMyc-induced apoptosis, does it also block cMyc-induced proliferation? This is important to determine because the synergy between cMyc and Bcl-2 can be easily rationalised only if Bcl-2 blocks the apoptotic function of cMyc but leaves its mitogenic activity unaffected. For this reason, we examined the mitotic rate of Rat-1/Myc-ER cells, either with or without Bcl-2 expression, proliferating in low serum under the influence of β -oestradiol-activated cMyc. In such an experiment, a proportion of the cells without Bcl-2 die before undergoing division, as previously described (see Examples 1 and 2) and thus cannot be scored. We therefore excluded such cells from our analysis and followed 100 of the remaining cells, chosen at random, through their first divisions. The results (Figure 11) show that mitotic rates of cells are similar irrespective of Bcl-2 expression, demonstrating that Bcl-2 expression does not prevent cell proliferation. Bcl-2 does not, therefore, inhibit all cMyc functions but specifically blocks cMyc-induced apoptosis.

Deregulated cMyc expression induces the rapid onset of apoptosis in cells arrested by a variety of cytostatic and cytotoxic drugs (see Examples 1 and 2). Many primary tumours also respond to such drugs by initiating apoptosis (Lennon *et al*, 1990) and, because myc gene activation is so

widespread in human tumours (Spencer & Groudine, 1991), we have suggested this is also cMyc-induced (see Examples 1 and 2). If correct, this notion implies that lesions in the apoptotic pathway, such as Bcl-2 activation, might contribute towards drug resistance in tumours. We

5 therefore investigated the effect of Bcl-2 expression on cMyc-induced apoptosis in Rat-1/Myc-ER cells arrested either with thymidine, which blocks in S-phase, or with the epipodophyllatoxin etoposide (VP16), a topo-isomerase II inhibitor that arrests cells in S/G2 and is frequently used in cancer chemotherapy (Liu, 1989). In both cases, Bcl-2 expression

10 substantially delays and reduces cMyc-induced apoptosis (Figure 12), effectively increasing the resistance of cells to either drug. In neither case did Bcl-2 expression overcome the cytostatic effect of either drug (data not shown). Deregulated expression of *c-myc* in tumour cells is so common as to suggest that its acquisition may be essential during carcinogenesis.

15 The potency with which *c-myc* induces apoptosis, however, suggests that deregulated *c-myc* expression by itself is likely to be lethal because it kills any cell that encounters growth-limiting conditions, an almost invariable outcome *in vivo*. In such a situation, any additional mutation that blocks apoptosis will strongly promote survival of the affected cell and so allow

20 its continued proliferation, mutation and carcinogenic evolution. Our results demonstrate that acquisition of constitutive Bcl-2 expression is an example of just such a mutation. Bcl-2 mitigates the apoptotic effects of deregulated cMyc expression without affecting its ability to promote continuous cell growth, so providing a mechanistic basis for the oncogenic

25 synergy between these two proto-oncogenes. Interestingly, the interaction between *c-myc* and Bcl-2 differs from the classical form of oncogene cooperation observed between cMyc and activated RAS (Land *et al*, 1983) in that, although cMyc/Bcl-2 fibroblasts exhibit continuous proliferation in the absence of mitogens, they neither appear morphologically

30 transformed nor form foci in monolayer culture (Reed *et al*, 1990 and see

Figure 9). The interaction between cMyc and Bcl-2 thus represents a novel type of oncogene cooperation undetectable by classical transformation focus assays. Further characterisation of the growth and death properties of cells containing various combinations of activated c-
5 *myc*, *ras* and *bcl-2* oncogenes should provide insights as to how the various attributes of these three classes of oncogene interact. The emergence of drug-resistant mutations in tumours reflects the fact that carcinogenesis is a continuous evolutionary process, often exacerbated by the increased genetic instability of cancer cells. Our results indicate that,
10 just as activated cMyc expression may be important in determining the initial sensitivity of tumours to drugs, so Bcl-2 deregulation, by blocking programmed cell death, may lead to drug resistance. This is a mechanism of drug resistance distinct from amplification and multi-drug resistance that have been previously described (Borst, 1991). The interaction
15 between cMyc and Bcl-2 thus represents a novel paradigm for oncogene cooperation which has implications both for the genesis and for the progression of neoplastic disease.

Figure 9. 9A: Determination of expression of Bcl-2a protein by
20 immunoblotting Lysates from control Rat-1/*myc*-ER cells (tracks 1 and 2) and a representative Rat-1/*myc*-ER/Bcl-2 clone (clone 5) (tracks 3 and 4) were fractionated on a 15% SDS polyacrylamide gel, blotted onto nitrocellulose paper and probed with either mouse (Bcl-2/100 - tracks 1 and 3) or a hamster (6C8 - tracks 2 and 4) anti-Bcl-2 monoclonal
25 antibodies. 9B: Constitutive Bcl-2 expression prevents cMyc-induced apoptosis in serum-deprived Rat-1 fibroblasts.

Various Rat-1-derived cell lines were transferred into medium containing 0.05% foetal calf serum either with or without 2mM β -oestradiol and
30 observed after 48 hours. 1 - Rat-1/Myc-ER, no β -oestradiol (cMyc

inactive); 2 - Rat-1/Myc-ER plus β -oestradiol (cMyc active); 3 - Rat-1/Myc-ER/Bcl-2, no β -oestradiol; 4 - Rat-1/Myc-ER/Bcl-2, plus β -oestradiol.

5 Methods

Production and maintenance of Rat-1 fibroblasts stably expressing the cMyc-ER fusion protein and the defective cMyc mutant protein $\Delta 106-143$ MycER have been described (see Examples 1 and 2). A
10 retrovirus vector directing constitutive expression of Bcl-2a was made by expressing a full length *bcl-2a* cDNA in pBabe puro (Morgenstern & Land, 1991). Infectious ecotropic Bcl-2/PURO virus was prepared from culture supernatants of WE cells (Morgenstern & Land, 1991), transfected with the Bcl-2/PURO vector and this was used to infect Rat-1/Myc-ER
15 cells (Morgenstern & Land, 1991). Infected clones were selected in $5\mu\text{g/ml}$ puromycin. Immunoblotting of bulk cultures of puromycin resistant cells was performed as described previously (Evan & Hancock, 1985) using either the Bcl-2/100 mouse monoclonal antibody (Pezella *et al.*, 1990) (a generous gift from Dr D.Y. Mason, Dept. Haematology, Oxford, UK) or the 6C8 hamster monoclonal antibody (Hockenbery *et al.*,
20 1990) (a generous gift from Dr S.J. Korsmeyer, Howard Hughes Medical Institute, Washington University, St Louis, USA).

Figure 10. Time-lapse cinemicroscopic quantitation of apoptosis in Rat-1 cells Rat-1 cells expressing either wild type cMyc-ER or the defective
25 cMyc mutant $\Delta 106-143$ fused to ER, and either with or without Bcl-2, were growth arrested by culture in 0.05% FCS for 48 hours. cMyc was activated by addition of $2\mu\text{M}$ β -oestradiol to the culture medium and cells observed by time-lapse cinemicroscopy. At no time did the cells reach confluence. Apoptotic cell deaths were scored as described (see Examples
30 1 and 2) and cumulative deaths plotted against time. Each field started

with 95 cells and images were taken at a rate of 12 frames per hour.

Figure 11. Time-lapse cinemicroscopic analysis of the effect of Bcl-2 expression on Myc-induced proliferation of Rat-1 cells Rat-1/Myc-ER and Rat-1/Myc-ER/Bcl-2 cells were cultured in 0.05% FCS containing 2 μ M β -oestradiol and mitotic events observed by time-lapse cinemicroscopy. Cells that underwent apoptosis prior to division were excluded from the analysis. Of the remaining cells, 95 were randomly picked and their fates followed. 12 frames were recorded per hour. Only first divisions in each lineage were counted in order to permit comparability between experiments. Cumulative divisions are shown plotted against time.

Figure 12. Constitutive Bcl-2 expression protects Rat-1/Myc cells from thymidine and VP16/etoposide-induced apoptotic cell death A - Thymidine block. Exponentially growing Rat-1/Myc-ER and Rat-1/Myc-ER/Bcl-2 cells were arrested in S phase by addition of 2mM thymidine to the growth medium for a period of 24 hours, exactly as previously described (see Examples 1 and 2). cMyc was then activated by addition of β -oestradiol to a final concentration of 2 μ M and the cells monitored by time-lapse cinemicroscopy at a rate of 12 frames/hour. Cumulative apoptotic deaths are shown plotted against time. One hundred of each cell type were examined. B - Etoposide/VP16 block. Exponentially growing Rat-1/Myc-ER and Rat-1/Myc-ER/Bcl-2 cells in 10% FCS were incubated for 24 hours with 0.1mM etoposide/VP16 (Sigma). cMyc was then activated by addition of β -oestradiol to a final concentration of 2 μ M and the cells monitored by time-lapse cinemicroscopy at a rate of 12 frames/hour. Cumulative apoptotic deaths are shown plotted against time. One hundred of each cell type were examined.

Example 4: Antisense inhibition of Bcl-2.

Several studies have employed antisense oligonucleotide strategies to block expression of Bcl-2 in lymphoid and fibroblastic cells (Reed *et al*, 1990a; 5 Reed *et al*, 1990b; Reed *et al*, 1991). These have used either antisense *bcl-2* sequences driven from the promoter of a transfected antisense gene (Reed *et al*, 1990a) or antisense oligonucleotides (Reed *et al*, 1990b; Reed *et al*, 1991) and have, in general, demonstrated a reduced proliferative capacity for the antisense-treated cells. Two of the studies used tumour 10 cells of the lymphoid lineage as their test system, one examines the effects of Bcl-2 expression in NIH 3T3 fibroblasts. Survival of lymphoid cells was also enhanced.

Our studies disclosed herein, on the potent apoptotic effects of the *c-myc* 15 oncogene, a proto-oncogene pervasively activated in human tumours, led us to propose that during carcinogenesis human tumours acquire an enhanced tendency to undergo apoptosis by virtue of their activated expression of *c-myc* (see Examples 1 and 2). Thus, we concluded that lesions in the apoptotic pathway were likely to be strongly selected for 20 during tumourigenic progression and during acquisition of resistance to cytotoxic drugs (see Example 3). By this argument, correction of any lesions or blockages in the apoptotic pathway of tumour cells would, at very least, render them once again drug-sensitive and might, in addition, be sufficient to elicit spontaneous remission. Thus, the activated *bcl-2* 25 gene, as the only currently known oncogene with anti-apoptotic activity, is an obvious target for inhibition. Moreover, although initially discovered in B lymphoid tumours, *bcl-2* is quite widely expressed (Hockenbery *et al*, 1991) and, in addition, active as an anti-apoptotic agent even in cells in which it is not normally expressed (Example 3). 30 Thus, we believe that *bcl-2* has a role to play in non-lymphoid

carcinogenesis.

Oligonucleotides and derivatives for inhibition of Bcl-2 expression.

Antisense sequences used to block *bcl-2* expression cover the initiation
5 codon of the *bcl-2* open reading frame. Oligonucleotides, typically 18-21
bases in length and synthesised as conventional oligodeoxynucleotides or
as phosphorothioate or phosphoramidate derivatives, are useful.
Phosphorothioate and phosphoramidate oligodeoxynucleotides derivatives
exhibit higher stability *in vitro* and *in vivo* (Agrawal *et al*, 1988; Campbell
10 *et al*, 1990; Matsukura *et al*, 1987; Stein *et al*, 1988; Woolf *et al*, 1990)
but possess lower T_m (ie dissociate from their complementary sequences
at lower temperatures) relative to their normal counterparts (Stein *et al*,
1988) and may thus be less effective on a molar basis. Alternatively,
bcl-2 antisense 2'-O-methylribonucleotide is employed: these RNA
15 oligonucleotides have a higher T_m with their complementary sequences
compared to deoxyribonucleotides, and have significantly higher stability
both *in vitro* and *in vivo* (Beijer *et al*, 1990; Iribarren *et al*, 1990; Lamond
et al, 1990).

20 Optimal sequences for antisense inhibition are sequences that span the
initiation codon of the human *bcl-2* open reading frame. The sequence
shown below is especially effective as it contains a significant proportion
of G \equiv C base pairs which are more stable at physiological temperatures
than A=T base pairs.

25

The antisense 22mer sequence corresponding to the sense target sequence
spanning the initiation codon of *bcl-2* is:

5'-C CCA GCG TGC GCC ATC CTT CCC-3' (SEQ3) or 5'-C CCA
30 GCG UGC GCC AUC CUU CCC-3' (SEQ4)

Other suitable antisense oligonucleotides or constructs may be designed based on the sequence of the *bcl-2* gene (Figure 13).

Delivery of antisense oligonucleotides.

5

Effective delivery of *bcl-2* antisense oligonucleotides *in vivo* is via liposomes (Loke *et al*, 1988; 1989). These are targeted in a variety of ways. For example, by coating the liposomes with antibodies specific for the tumour cells. The significant advantage of anti-*bcl-2* strategies is that
10 inhibiting *bcl-2* is not especially likely to be toxic to bystanding cells even if it enters them. This is because most normal cells are prevented from undergoing apoptosis by a variety of cytokine mechanisms. It is specifically the tumour cell that needs to avoid apoptosis in order to survive and grow and thereby is dependent upon continuous *bcl-2*
15 expression.

Example 5: Expression of Bcl-2 in hybridomas to enhance growth and productivity.

20 Bcl-2 is expressed in hybridomas in order to enhance their resistance to adverse culture conditions and increase their cloning efficiency and survival at high density. The *bcl-2* gene is introduced either into the parent lines prior to fusion with lymphocytes, in which case all resultant hybridomas would be expected to express Bcl-2, or into existing
25 hybridomas in order to potentiate their growth *in vitro*. Expression of the *bcl-2* gene is driven either from the MoMuLV LTR or from the CMV promoter. Bcl-2 is expressed in the hybridoma parent lines currently used, namely SP2/0, X63, NS1 and NS0. We have determined that none of these cell lines normally expresses endogenous Bcl-2 protein and
30 therefore its introduction will have useful phenotypic consequences in

- promoting survival and hardiness. Bcl-2 is introduced either by transfection or by retrovirus infection with appropriate Bcl-2-expressing constructs. Parent hybridomas expressing Bcl-2 are initially selected using whatever drug resistance is present in the Bcl-2 construct (ie G418 at 500 $\mu\text{g/ml}$, puromycin at 3-5 $\mu\text{g/ml}$, hygromycin at 200 $\mu\text{g/ml}$). Drug-resistant clones are isolated by standard ring cloning and assessed for Bcl-2 expression by immunocytochemical and immunoblotting techniques using Bcl-2-specific antibodies (see Example 3). The Bcl-2-expressing parent myeloma cells are used for fusion to immune lymphocytes exactly as in standard fusion protocols. The only difference is the continuous presence of the drug used as the selectable marker in the Bcl-2 construct. This ensures that the exogenous *bcl-2* gene is not lost during the course of the procedure.
- 15 The benefits of expressing Bcl-2 in parental myeloma lines and hence in resultant hybridomas are increased cloning efficiencies and resistance to the unavoidable adverse culture conditions (eg very low densities, presence of large numbers of dead cells, alkaline or acidic growth medium) that occur during the early phases post-fusion, and reduced loss of antibody-positive hybridomas during early cloning. The resulting Bcl-2-expressing hybridomas produce higher yields of antibody, exhibit less capriciousness in the growth of the secreting hybridoma in culture, show greater resistance to transient or long term adverse culture conditions, grow to and survive at higher densities *in vitro*, show more consistent growth in culture over time and from occasion to occasion, and have decreased requirements for foetal calf serum and expensive cytokine additives.
- 20
- 25
- 30 Introduction of the *bcl-2* gene into existing hybridomas is carried out either by transfection or retrovirus infection with appropriate drug-selectable *bcl-2* constructs essentially as described above for the parental

myeloma lines. The transfected hybridomas will be continuously maintained under this drug selection to prevent loss of the *bcl-2* gene. Any commercially important hybridoma should profit from introduction of Bcl-2 and consequent invigoration.

5

Status of myeloma parent lines expressing Bcl-2.

Hybridomas have a significant tendency to undergo apoptotic death *en masse* in response to transient sub-optimal culture conditions such as growth to high density, exhaustion of serum or other growth factors or excessive dilution (for example during single cell cloning). We have established that the myeloma/hybridoma cell lines NS1, NS0 and SP2/0 commonly used in hybridoma fusion experiments express essentially no *bcl-2* mRNA or protein and thus do not benefit from any protective properties derived from this gene. Accordingly, the introduction of *bcl-2* expression promotes survival of these cells. An exogenous human *bcl-2* gene expressed from a constitutive MoMuLV LTR promoter has been introduced into stock NS1 cells by retrovirus infection and several parameters of growth and resistance to insult assessed.

20

The *bcl-2* ORF has been cloned into a retroviral expression vector containing a moloney murine leukaemia virus promoter and a selectable puromycin resistance marker. After passage through the Ψ 2 packaging cell line the ecotropic *bcl-2*/PURO retrovirus is used to infect mouse or rat hybridomas or parental fusion partner myelomas (eg NS1, NS0, SP2/0). Positive clones are selected under 3-5 μ g/ml puromycin selection and tested for *bcl-2* expression by immunocytochemical staining with an anti-*bcl-2* peptide polyclonal antibody.

30 Compared with their non-*bcl-2*-expressing control counterparts, NS1/*bcl-2*

cells grow to an approximately 2 fold higher density in culture and exhibit extended survival under high density conditions. NS1/*bcl-2* cells also survive better in conditions of low serum and show higher cloning efficiencies after limiting dilution. Thus, expression of *bcl-2* confers
5 significant protective effects on the NS1 parent. Moreover, these results were obtained with primary pools of infected cells and contain clones expressing a range of levels of *bcl-2*. It is likely that the protective effects of *bcl-2* are dose-dependent. Thus, clones of NS1/*bcl-2* cells that express
10 higher levels of *bcl-2* cells may exhibit even greater resistance to cell death.

We have established that: (1) NS0, NS-1 and SP2/0 parent myeloma lines do not express *bcl-2* mRNA or Bcl-2 protein; (2) NS-1 expressing Bcl-2 or Bcl-2 and c-Myc exhibit more sustained growth and survival in low
15 serum (0% or 0.5%) and greater viability for longer at maximum densities. They also clone at higher efficiencies; and (3) the results are significant and can probably be bettered using individual clones selected to express higher levels of Bcl-2.

20 The effect of *bcl-2* expression on myeloma parent NS1 survival at high density is shown in Figures 14 and NS1 survival in 0.5% foetal calf serum (FCS) in Figure 15.

Figure 14 shows the results of introducing a constitutively active human
25 *bcl-2 α* gene into NS1 myeloma cells using the pBabe PURO retrovirus vector. *bcl-2* expression is driven from the retrovirus LTR promoter and puromycin resistance from the SV40 early promoter. Puromycin-resistant pools of NS1 cells that express Bcl-2 α protein and appropriate Bcl-2
controls were seeded at a density of 5×10^5 cells/ml in complete RPMI
30 medium + 10% foetal calf serum. Equivalent triplicate aliquots were

taken from the cultures every two days and the percentage cell viability measured by trypan blue dye exclusion (top) and live cell numbers assessed (bottom).

- 5 Figure 15 shows the results of introducing a constitutively active human *bcl-2* α gene into NS1 myeloma cells using the pBabe PURO retrovirus vector. *bcl-2* expression is driven from the retrovirus LTR promoter and puromycin resistance from the SV40 early promoter. Puromycin-resistant pools of NS1 cells that express Bcl-2 α protein and appropriate Bcl-2
- 10 controls were seeded at a density of 5×10^5 cells/ml in complete RPMI medium + 0.5% foetal calf serum. Equivalent triplicate aliquots were taken from the cultures every two days and numbers of live cells assessed by trypan blue dye exclusion (top) and percentage cell viability estimated (bottom).

15

Example 6: Stabilisation of other cell types.

- The efficacy of blocking *c-myc*-induced cell death in immortalised fibroblasts in which *bcl-2* is not naturally expressed implies that the
- 20 survival-promoting effects of *bcl-2* are not cell type-specific. Therefore *bcl-2* is introduced into a variety of cell lines used in the production of biopharmaceutical reagents as a way of increasing resistance of the appropriate cells to death as a consequence of overgrowth, depleted culture conditions, or as a consequence of the toxic effects of the reagent
- 25 they are producing. Mouse and rat cells may be infected with the murine ecotropic virus as described above and selected in puromycin. Human cells may be infected either with an amphotropic version of the *bcl-2*/PURO virus (ie that can infect human cells) or by transfection using lipofectin, calcium phosphate precipitation or electroporation (Sambrook
- 30 *et al*, 1989), as convenient and best suited to the respective cell type

concerned.

Using the methods described in Example 5, *bcl-2* expression is introduced into a range of cell lines. This may be useful in deriving variants of existing cell lines that are resistant to the vicissitudes of culture conditions or in cell types that spontaneously undergo apoptosis (eg cells derived from the embryonic central nervous system, lymphoid cells, haematopoietic cells) and in promoting establishment of cell lines from normal, dysplastic and neoplastic tissues without the need for recondite and/or costly growth factors.

Example 7: Assay systems for modulators of apoptosis.

Two kinds of modulator of apoptosis are of interest - agonists (inducers) and antagonists (blockers). Screening for antagonists of apoptosis requires a reproducible, easy and consistent system for inducing apoptosis in target cells. Genes, cytokines and chemicals that block apoptosis will yield surviving cells whereas control cells will all die.

The requirements for an assay system for anti-apoptotic agents can be summarised as follows:

- (1) An indicator cell line that can be induced to undergo apoptosis. The cell line should preferably be immortal, fast growing and easy to maintain.
- (2) A defined and reproducible system for inducing apoptosis, with a very low background of cell death in uninduced cells.
- (3) A rapid and easy quantitative assay for cell viability/cell death.

We have already described characterisation of an immortalised fibroblast

rat cell line (Rat-1/Myc-ER in Examples 1 and 2) in which apoptosis can be induced by activation of a conditional allele of the *c-myc* oncogene under specific defined culture conditions (ie absence of serum) by addition of β -oestradiol to the culture (Examples 1 and 2). We have also shown
5 that introduction of a specific anti-apoptotic gene (the human *bcl-2* gene) into such cells effectively blocks apoptosis and allows survival under conditions when control cells die (Example 3). Thus, we have fulfilled the criteria for a suitable indicator cell line and a controllable system for inducing apoptosis. We have also shown that genes exist that score as
10 apoptosis-blocking-genes in this assay.

Genes that interfere with apoptosis.

The Rat-1/Myc assay system allows the systematic screening of any
15 introduced gene (eg from tumour cells, normal cells, brain cells, drosphila cells etc) for survival-potentiating properties. This may enable rapid progress in defining the intracellular processes involved in regulating cell death and define new pharmacological targets.

20 Genes that block apoptosis are likely to be of two general kinds. (1) those, like *bcl-2*, that block apoptosis when inappropriately expressed (ie become active following regulatory mutations in their control elements) and (2) those that are normally involved in modulating the apoptotic pathway but only in response to the correct contextual signals.

25

Methodology to identify bcl-2-like genes.

Rat-1/Myc-ER cells are infected with MoMuLV. This integrates at random into the host cell genome and activates nearby genes. When
30 apoptosis is triggered in the host Rat-1/Myc-ER cells by withdrawing

- serum and adding 2 μ M β -oestradiol, all cells will die except those in which the infecting MoMuLV has activated expression of a survival gene. These cells are then propagated as clones. The clones are examined for common MoMuLV integration sites to identify likely target genes.
- 5 Cellular DNA adjacent to virus integration sites are cloned using an inverse PCR strategy (van Lohuizen *et al*, 1991) and the resultant genes cloned and sequenced. Antibodies may be raised against synthetic peptides comprising likely antigenic epitopes and the protein products of the survival genes characterised. As a first stage to this strategy, we have
- 10 identified 32 independent clones of MuLV-infected Rat-1/Myc-ER cells that are resistant to Myc-induced apoptosis but still express active Myc-ER. Thus, these clones appear to have acquired the ability to evade apoptosis through activation of novel genes.
- 15 *Methodology to identify anti-apoptotic mutated genes in tumours.*
- mRNA from aggressive and drug-insensitive tumours (ie tumours most likely to have acquired anti-apoptotic lesions) is randomly reverse transcribed and the resultant double stranded cDNAs are inserted into a
- 20 CMV expression vector. The cloned tumour sequences are then introduced into Rat-1/Myc-ER cells by electroporation or lipofection and cells carrying transfected sequences selected with neomycin by virtue of the selectable marker in the plasmid. Rat-1/Myc-ER cells will then be triggered to undergo apoptosis by withdrawing serum and adding 2 μ M β -
- 25 oestradiol. All cells will die except those which have acquired novel survival genes from the original tumour. Survival gene sequences will be directly isolated by PCR using primers flanking the plasmid cloning site in which they were introduced.

Cytokines that interfere with apoptosis.

We have shown that apoptosis in the Rat-1/Myc indicator assay system can be almost completely blocked by addition of specific cytokines to the medium for example IGF1 and PDGF AB. Thus various cytokines may be screened for their abilities to specifically block apoptosis. By transfecting into our Rat-1/Myc indicator cells various components of non-fibroblast signal transduction pathways (eg NGF receptor, IL-3 receptor) it may be possible to screen for the activity of cytokines not normally active in fibroblasts.

Small Molecular Weight Compounds that interfere with apoptosis.

This assay, that can measure inhibition of cell death and that is based on readily available and culturable immortalised Rat-1/Myc fibroblasts, may allow construction of a rapid through-put screen for small molecular weight compounds that block apoptosis. Rat-1/Myc cells are cultured as adherent cells in microtitre/Terasaki plates and then subjected to conditions that induce apoptosis (eg for the Myc-ER constructs, this would be by dropping serum levels and adding β -oestradiol) in the presence of small molecular weight compounds or broths. Most chemicals will be toxic or neutral to cells and all the cells in such culture wells will die. Cells in which apoptosis is blocked by a small molecular weight compound may be identified by the presence of live cells which can be detected by fluorescence (eg cleavage of added fluorescein diacetate), by incorporation of ^3H -thymidine, or by their continued growth and survival.

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Zetterberg, A. and Larsson, O. (1985) *Proc Natl Acad Sci USA* 82, 5365-9.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Imperial Cancer Research Technology Ltd
- (ii) TITLE OF INVENTION: Modified cells and method of treatment
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
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 - (B) STREET: St Mary's Court, St Mary's Gate
 - (C) CITY: Nottingham
 - (D) STATE: Nottinghamshire
 - (E) COUNTRY: United Kingdom
 - (F) ZIP: NG1 1LE
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Bassett, Richard S
 - (C) REFERENCE/DOCKET NUMBER: IMPF/P11528
- (ix) TELECOMMUNICATION INFORMATION:
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 - (B) TELEFAX: (0602)588122
 - (C) TELEX: 37540 Potter G

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 765 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (v) ORIGINAL SOURCE:
 - (A) ORGANISM: Homo sapiens
- (viii) POSITION IN GENOME:
 - (A) CHROMOSOME/SEGMENT: 18
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 31..747
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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1

5

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102

Tyr Asp Asn Arg Glu Ile Val Met Lys Tyr Ile His Tyr Lys Leu Ser
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Gln Arg Gly Tyr Glu Trp Asp Ala Gly Asp Val Gly Ala Ala Pro Pro
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GGG GCC GCC CCC GCA CCG GGC ATC TTC TCC TCC CAG CCC GGG CAC ACG
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294

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342

Val Pro Pro Val Val His Leu Ala Leu Arg Gln Ala Gly Asp Asp Phe
90 95 100

TCC CGC CGC TAC CGC GGC GAC TTC GCC GAG ATG TCC AGC CAG CTG CAC
390

Ser Arg Arg Tyr Arg Gly Asp Phe Ala Glu Met Ser Ser Gln Leu His
105 110 115 120

CTG ACG CCC TTC ACC GCG CGG GGA CGC TTT GCC ACG GTG GTG GAG GAG
438

Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala Thr Val Val Glu Glu
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CTC TTC AGG GAC GGG GTG AAC TGG GGG AGG ATT GTG GCC TTC TTT GAG
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TTC GGT GGG GTC ATG TGT GTG GAG AGC GTC AAC CGG GAG ATG TCG CCC
534

Phe Gly Gly Val Met Cys Val Glu Ser Val Asn Arg Glu Met Ser Pro
155 160 165

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582

Leu Val Asp Asn Ile Ala Leu Trp Met Thr Glu Tyr Leu Asn Arg His
170 175 180

CTG CAC ACC TGG ATC CAG GAT AAC GGA GGC TGG GAT GCC TTT GTG GAA
630

Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp Asp Ala Phe Val Glu
185 190 195 200

77

CTG TAC GGC CCC AGC ATG CGG CCT CTG TTT GAT TTC TCC TGG CTG TCT
678

Leu Tyr Gly Pro Ser Met Arg Pro Leu Phe Asp Phe Ser Trp Leu Ser
205 210 215

CTG AAG ACT CTG CTC AGT TTG GCC CTG GTG GGA GCT TGC ATC ACC CTG
726

Leu Lys Thr Leu Leu Ser Leu Ala Leu Val Gly Ala Cys Ile Thr Leu
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765

Gly Ala Tyr Leu Ser His Lys
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 239 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
20 25 30

Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
35 40 45

Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
50 55 60

Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
65 70 75 80

Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
85 90 95

Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
100 105 110

Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
115 120 125

Arg Phe Ala Thr Val Val Glu Leu Phe Arg Asp Gly Val Asn Trp
130 135 140

Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145 150 155 160

Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
165 170 175

Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
180 185 190

Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro
195 200 205

Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala
210 215 220

Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys
225 230 235

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCAGCGTGC GCCATCCTTC CC
22

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCAGCGUGC GCCAUCCUUC CC
22

CLAIMS

1. A method of treating tumour cells in a vertebrate, the method comprising introducing into the tumour cells means for inhibiting an inhibitor of *myc*-induced programmed cell death.
5
2. A method according to Claim 1 wherein the tumour cells are not lymphoma cells.
- 10 3. A method according to Claim 1 wherein the tumour cells are secondary tumour cells derived from a solid tumour.
4. A method according to any one of the preceding claims wherein the said means for inhibiting comprises means for preventing expression of an anti-oncogene or proto-oncogene.
15
5. A method according to Claim 4 wherein the anti-oncogene or proto-oncogene is *bcl-2*.
- 20 6. A method according to Claim 4 or 5 wherein the said means for inhibiting is antisense nucleic acid adapted to bind to the anti-oncogene or proto-oncogene or transcription products thereof.
7. A method according to Claim 6 wherein the said antisense nucleic acid is an antisense oligonucleotide.
25
8. A method according to Claim 6 wherein the said antisense nucleic acid is antisense RNA expressed from a DNA construct.
- 30 9. A method according to any one of the preceding claims wherein

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a coding sequence therein.

24. A method according to Claim 23 wherein the cell line is a hybridoma and the product is an antibody.
- 5
25. A product prepared by a method according to any one of Claims 22 to 24.
- 10
26. A DNA construct comprising the *bcl-2* coding sequence or an analogue thereof and means providing for constitutive expression thereof in a cell.
- 15
27. An assay for detecting whether a compound is involved in modulating apoptosis, the assay comprising a cell transformed with a DNA construct comprising a proto-oncogene coding sequence encoding a polypeptide which, when forcibly expressed or activated, induces apoptosis, and regulatory elements which allow transcription of the coding sequence.
- 20
28. An assay according to Claim 27 wherein the proto-oncogene is *myc*.
- 25
29. An assay according to Claim 27 or 28 wherein the regulatory elements include an inducible or repressible promoter.
- 30
30. An assay according to Claim 27 wherein the polypeptide encoded by the proto-oncogene is fused to a further polypeptide, and the fusion polypeptide is activated by addition of a small molecule.
- 30
31. An assay according to Claim 30 wherein the proto-oncogene is

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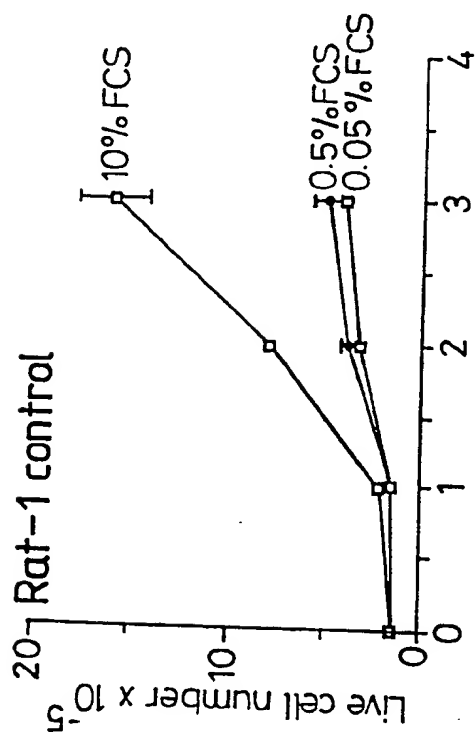


Fig. 1A

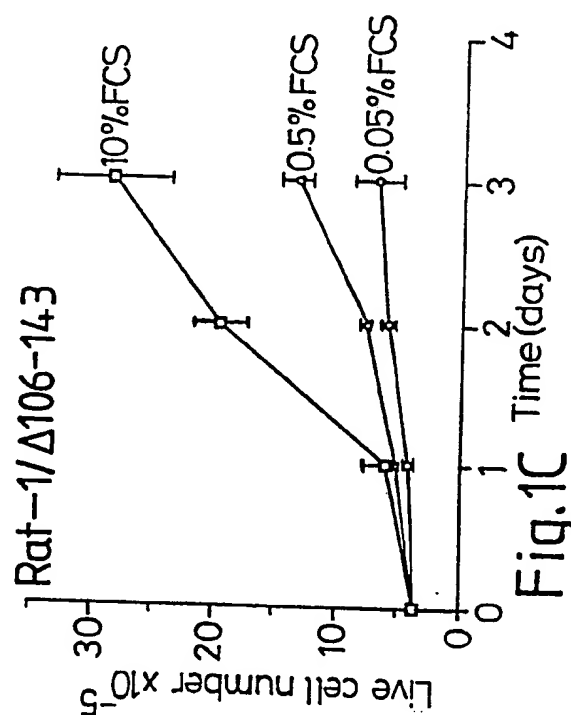


Fig. 1C

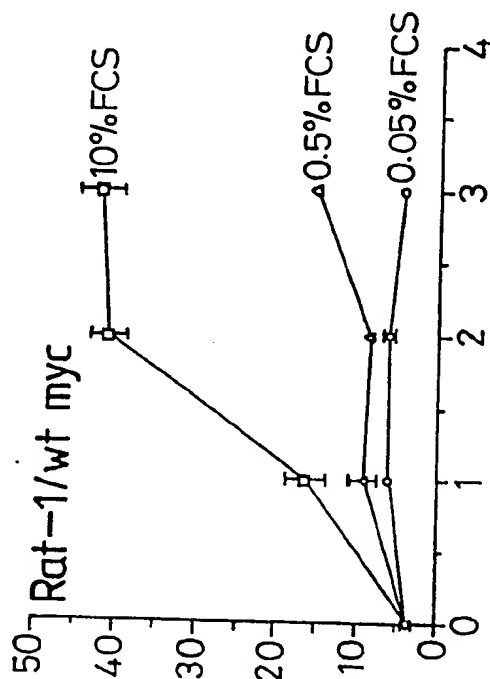


Fig. 1B

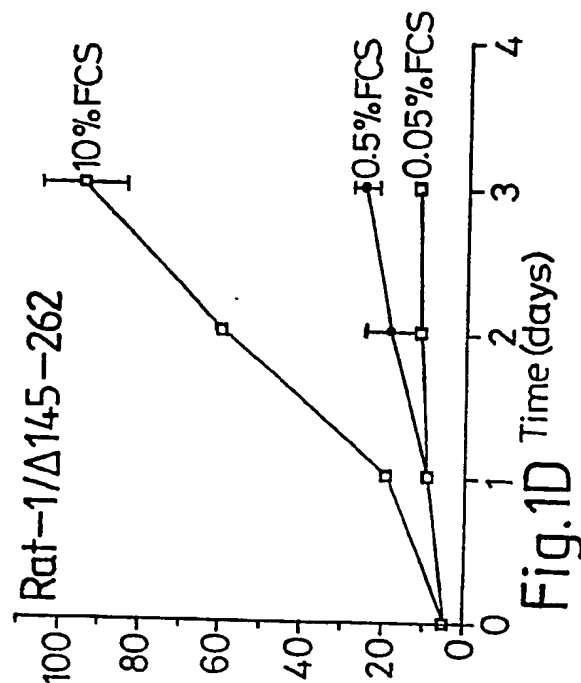


Fig. 1D

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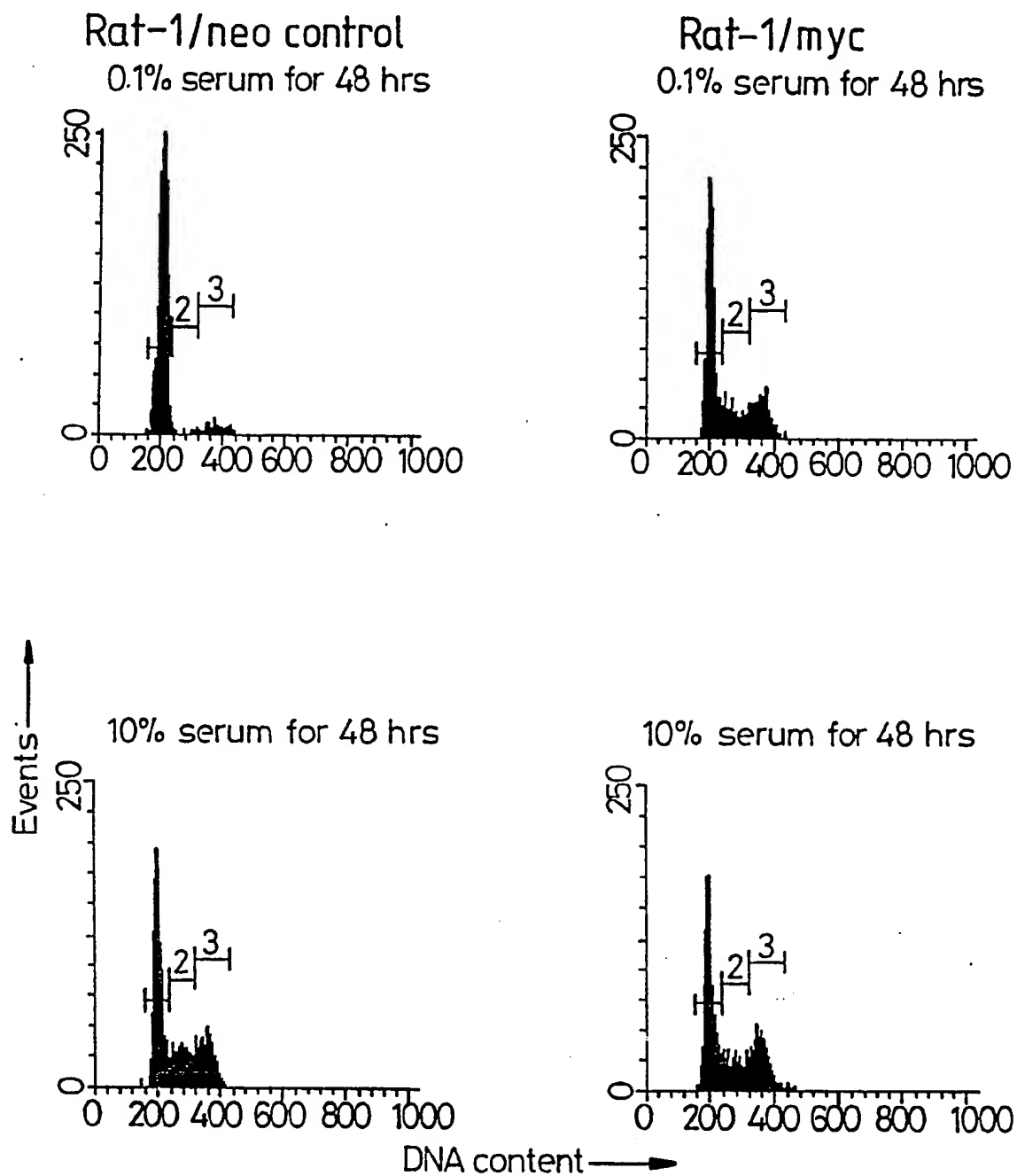


Fig. 2A

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Cell Line	Treatment (48 hrs)	% age in S- phase/hr
Rat-1/neo control	10% serum	50
	0.1% serum	3
Rat-1/myc	10% serum	41
	0.1% serum	45

Fig. 2B

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2

4

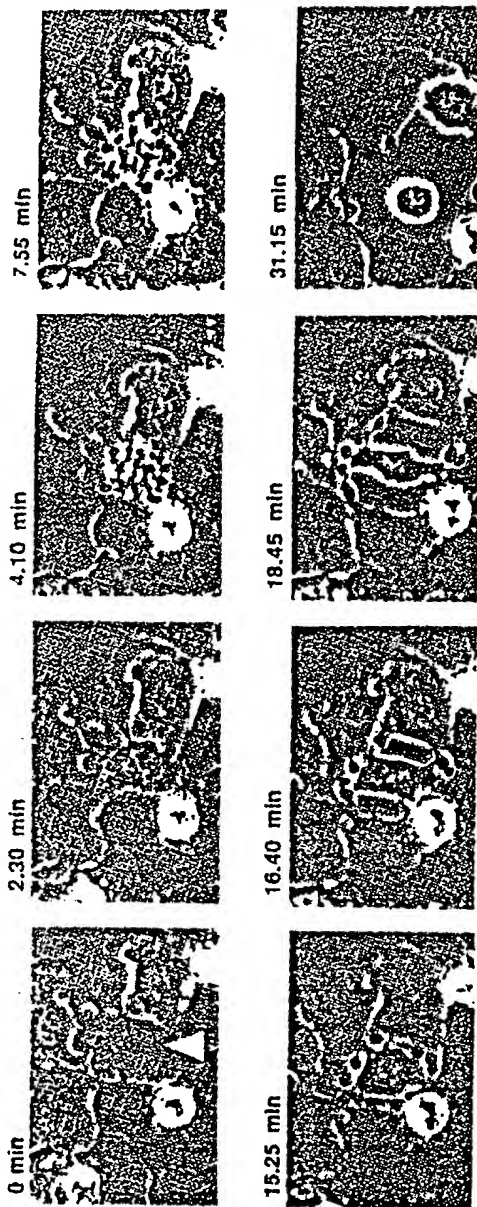


Fig. 3A

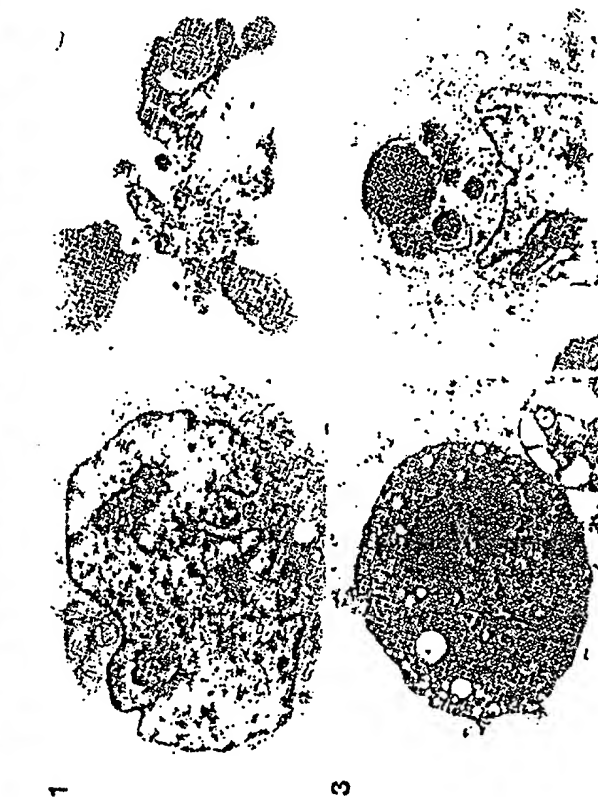


Fig. 3C

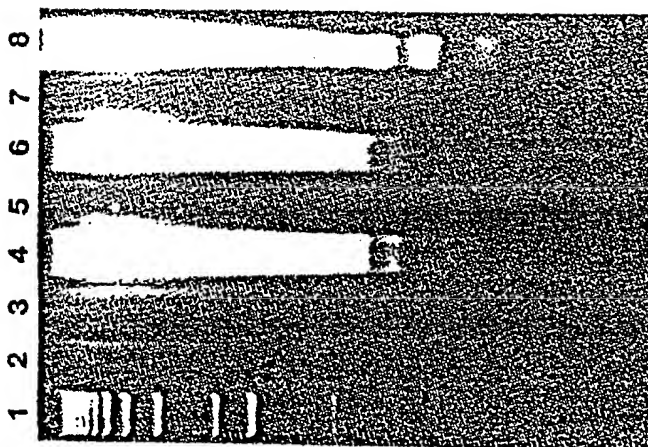


Fig. 3B

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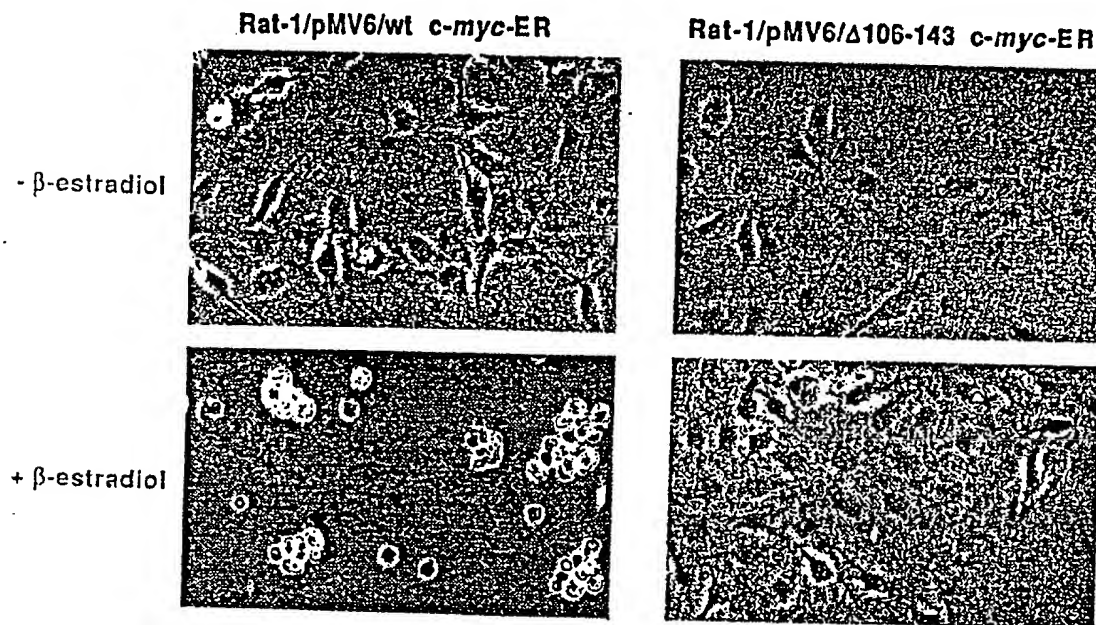


Fig. 4

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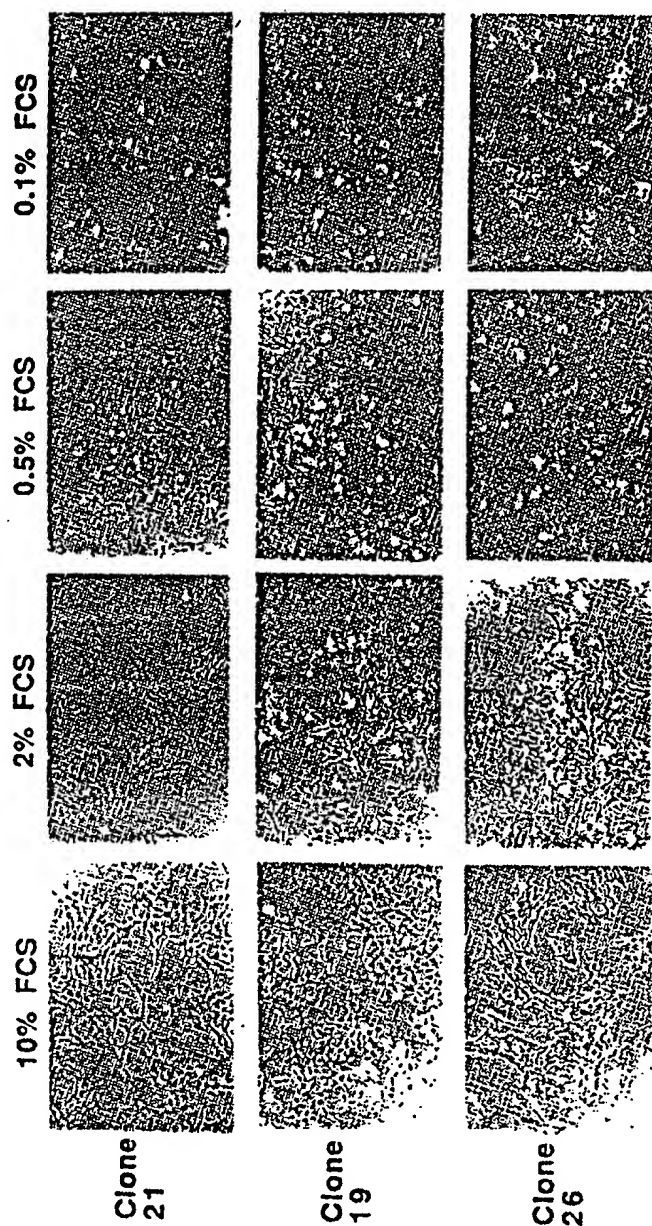


Fig. 5A

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CUMULATIVE CELL DEATHS

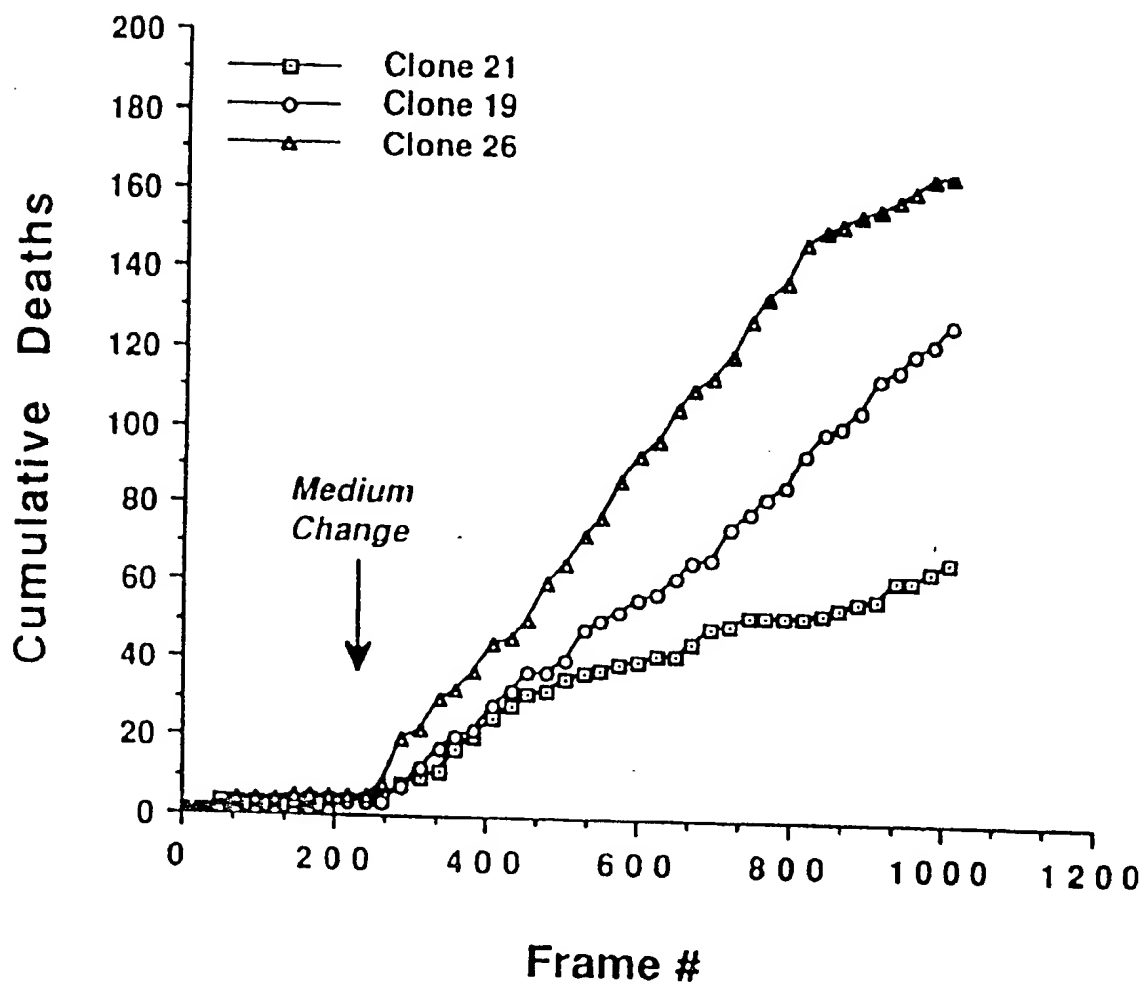


Fig. 5B

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LIVE CELL COUNT

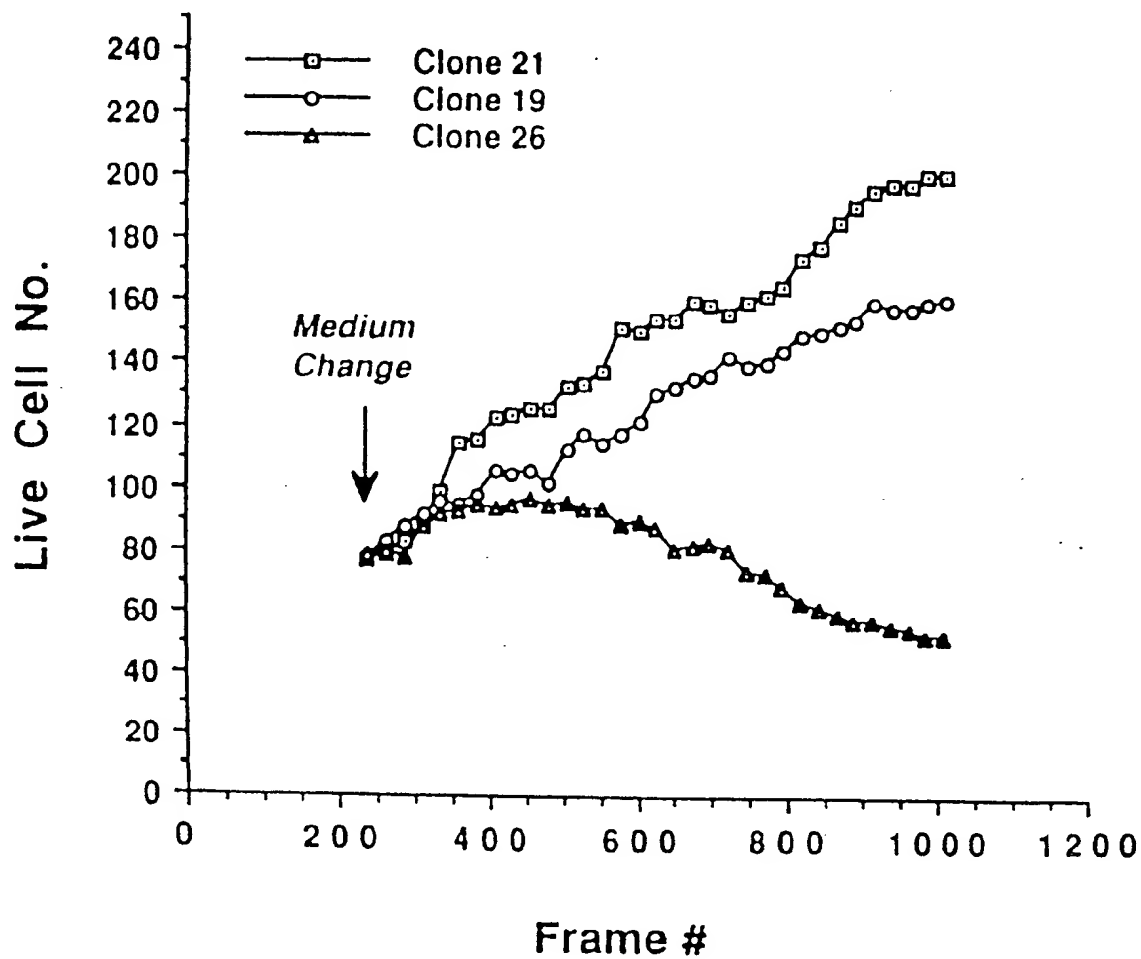
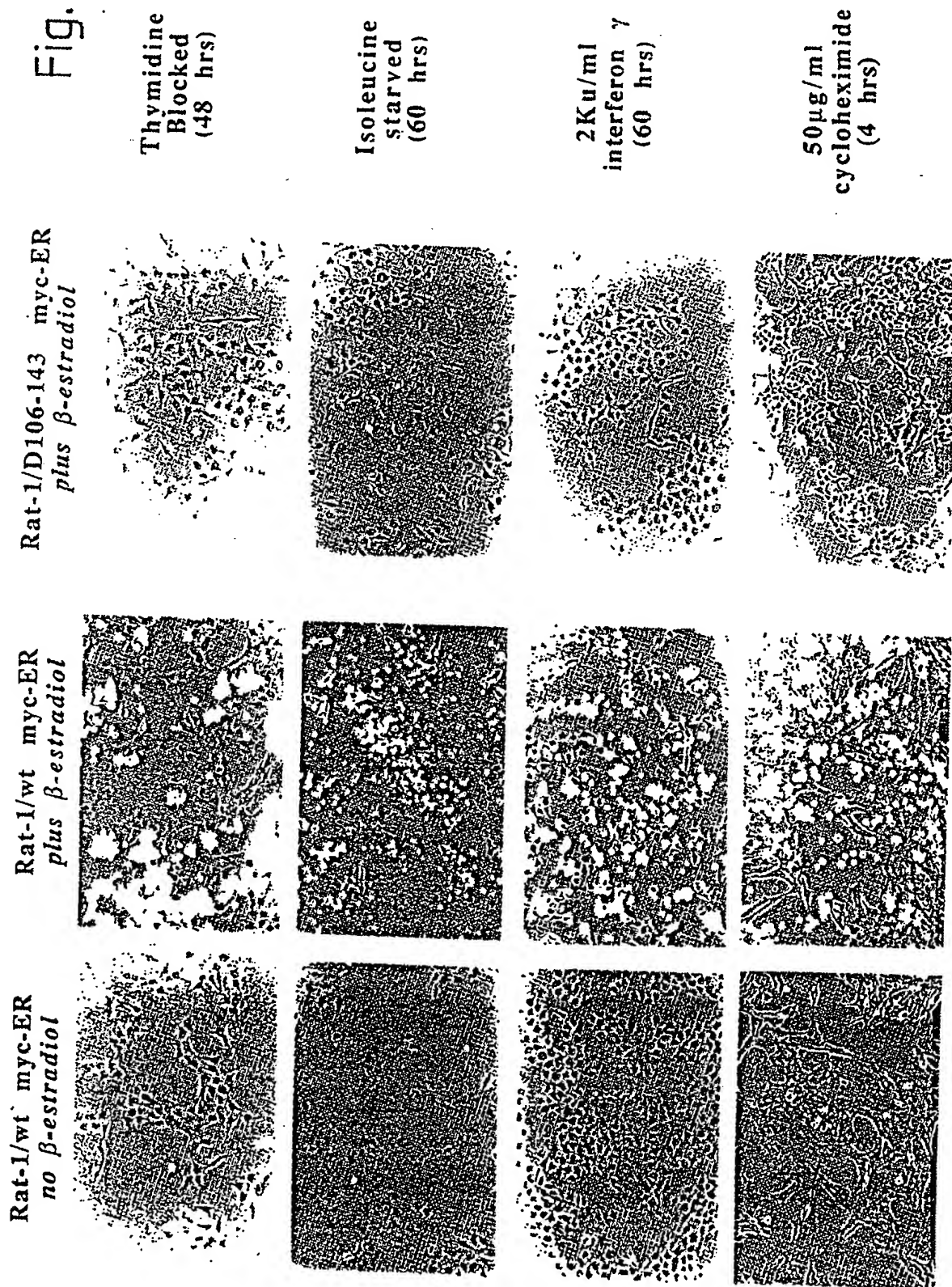


Fig. 5C

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Fig. 6



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Serum-starved (G0)

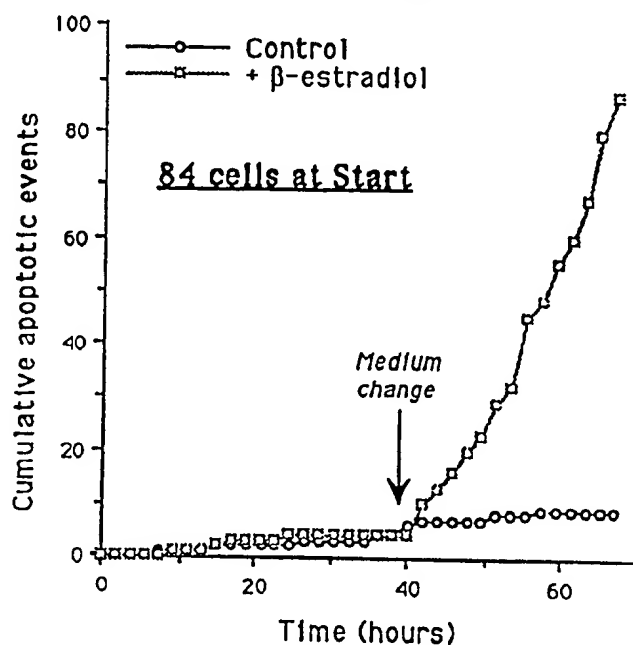


Fig. 7A

Isoleucine-starved

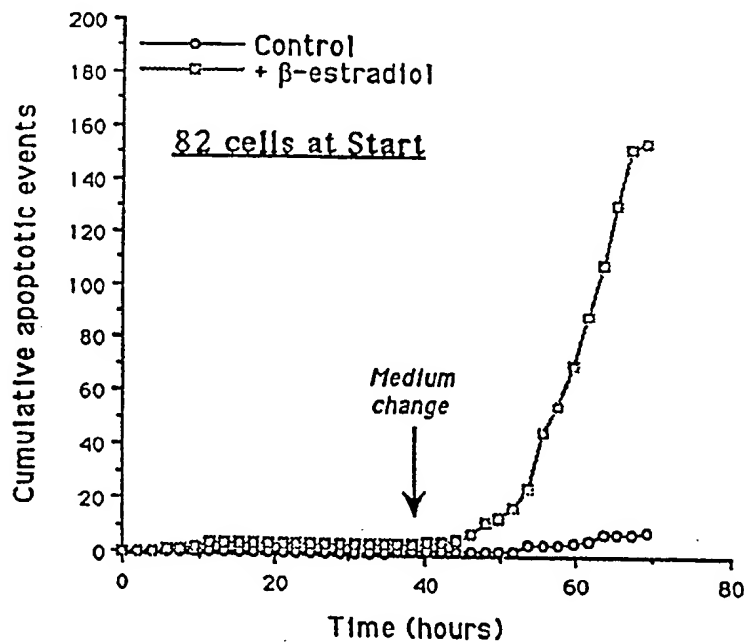


Fig. 7B

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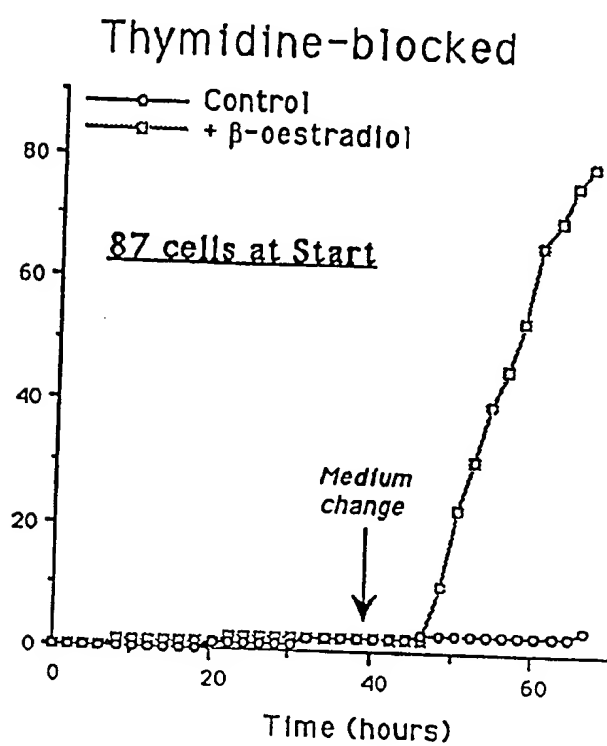


Fig. 7C

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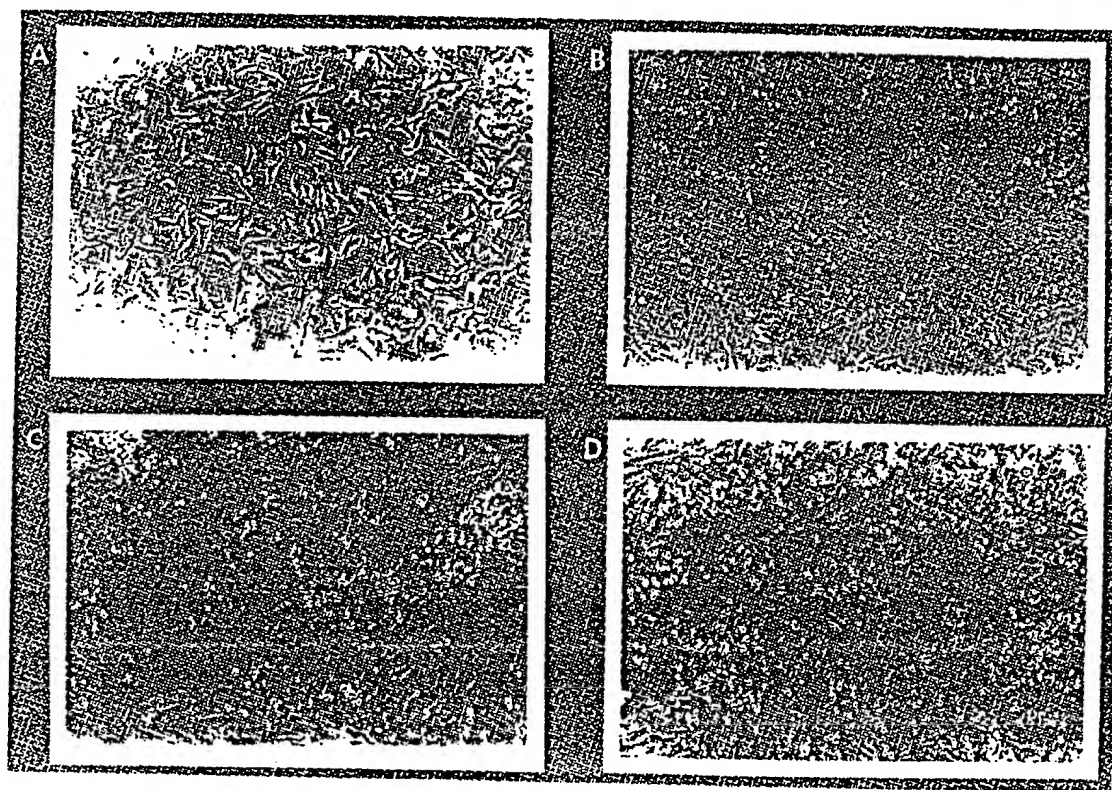


Fig. 8

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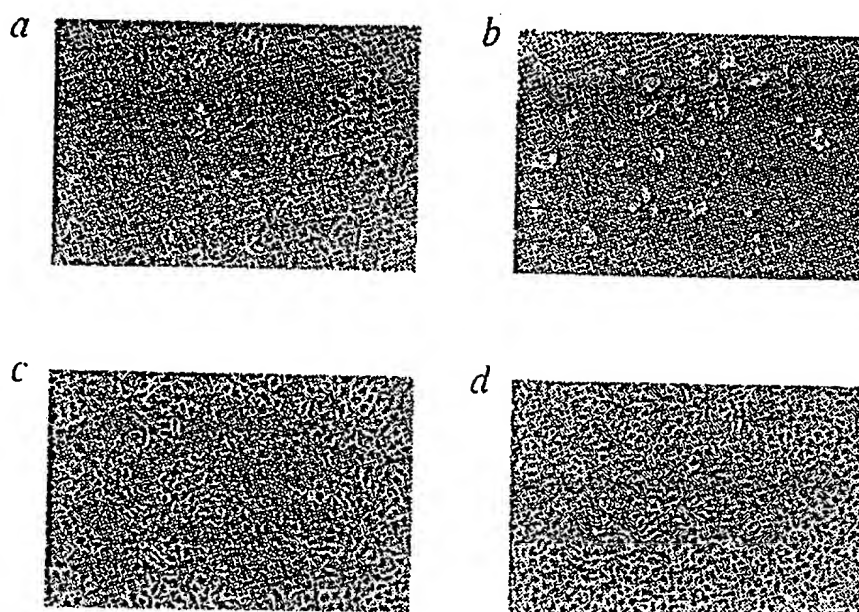


Fig. 9

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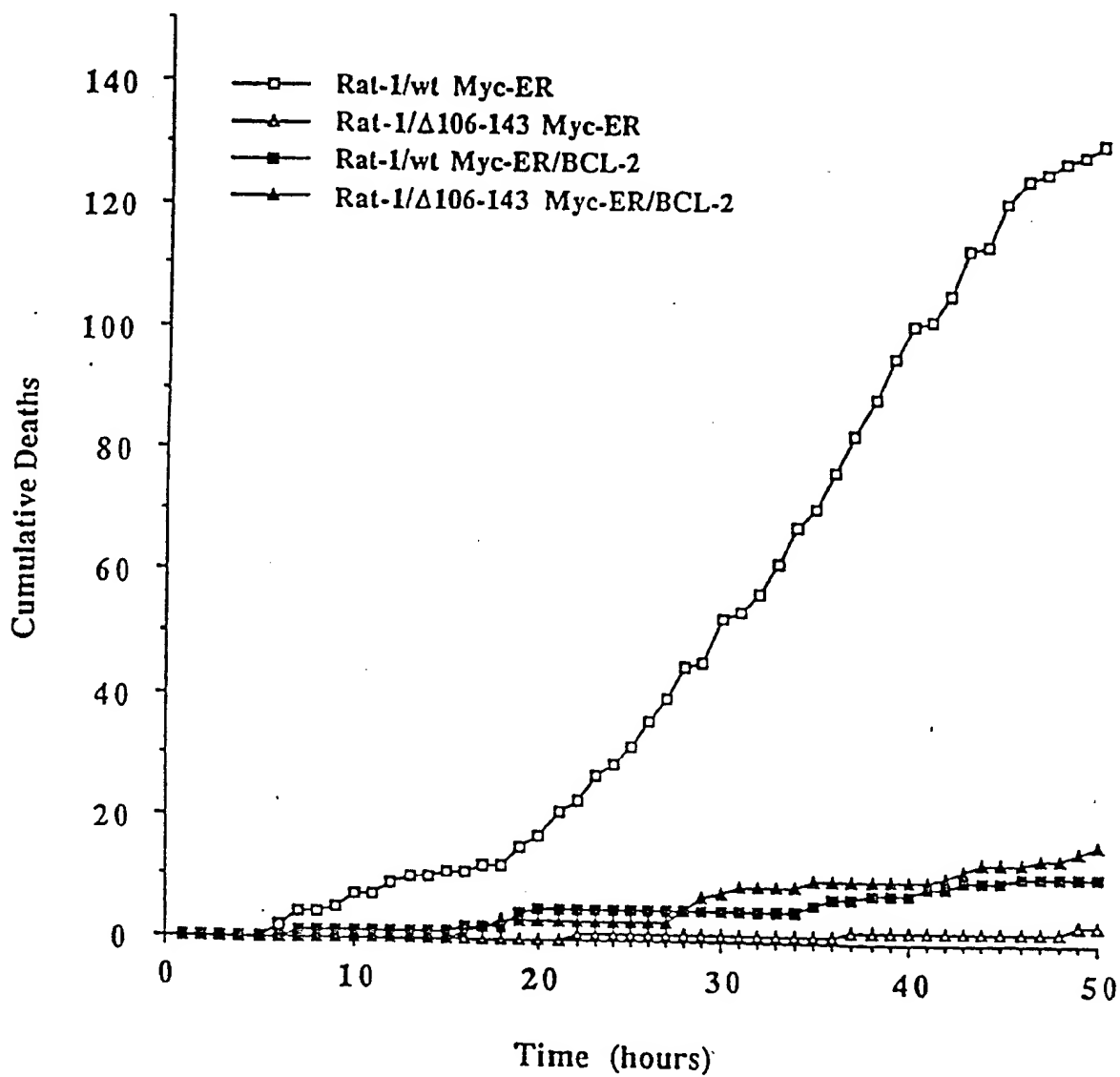


Fig. 10

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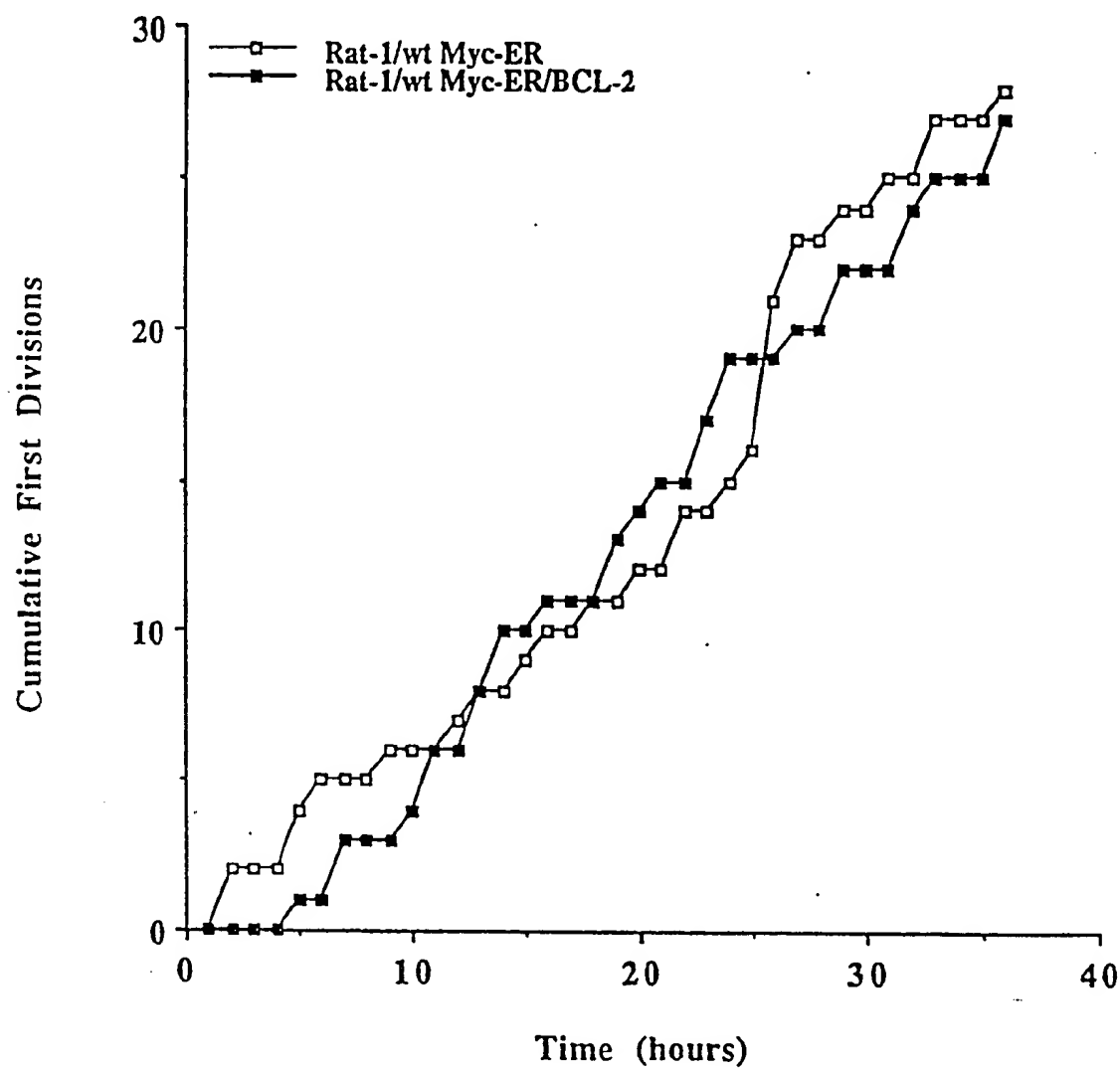
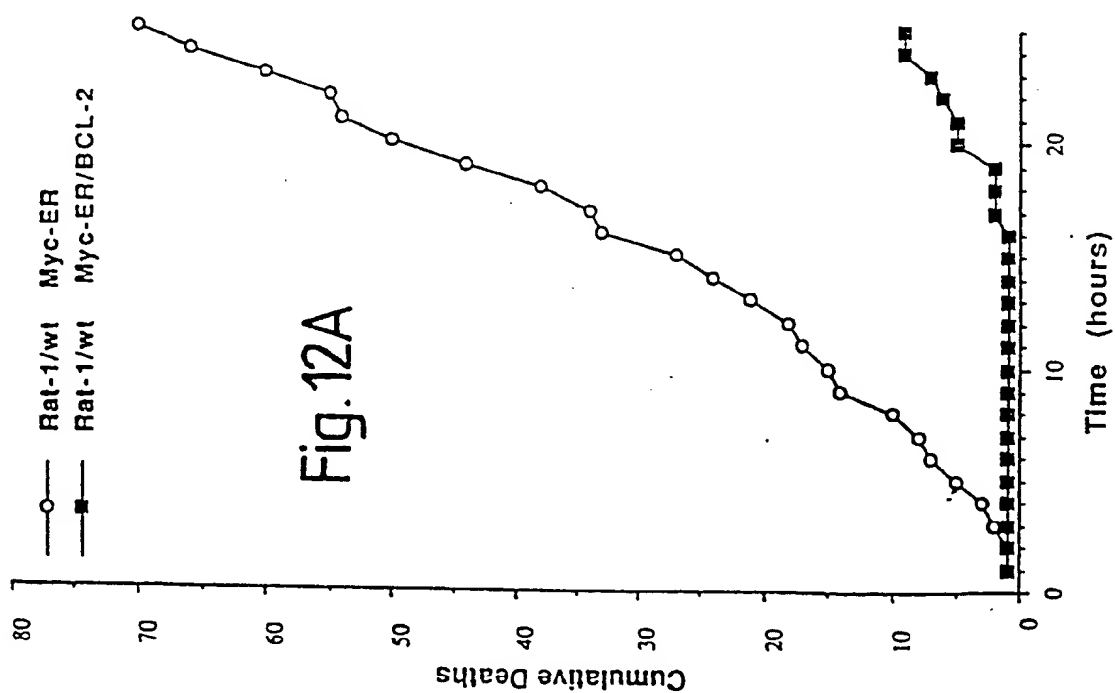
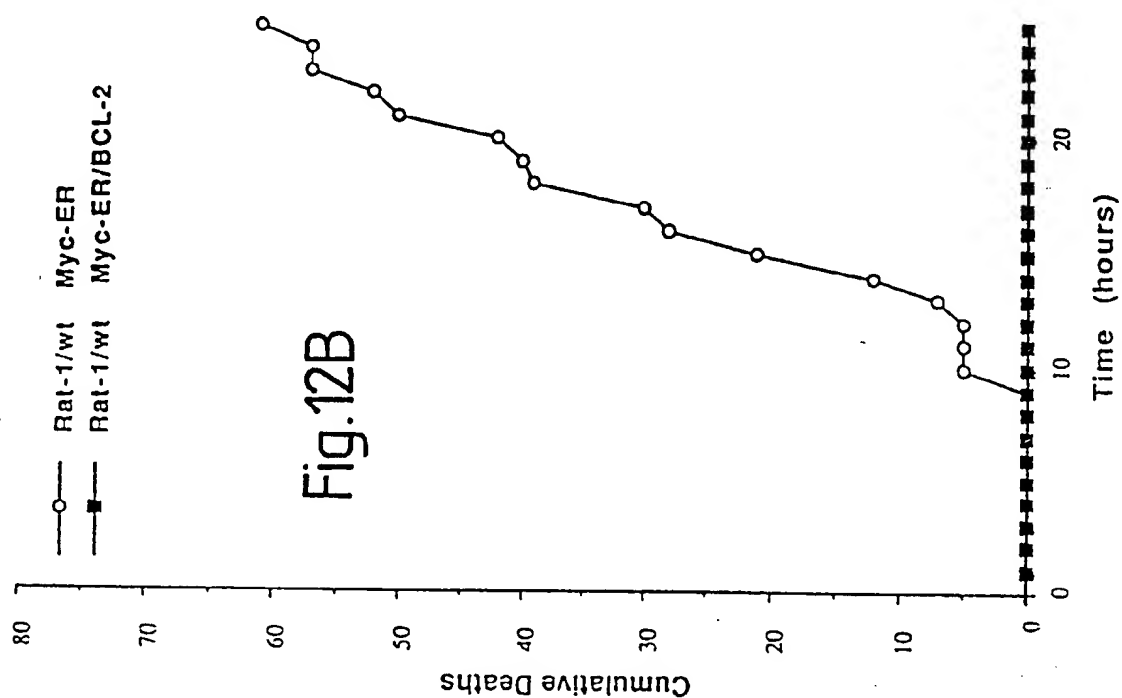


Fig.11

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Antisense sequence 3'-C CCT TCC TAC CGC GTG CGA CCC-5'

1479
*
TTG GCC CCC GTT GCT TTT CCT CTG GGA AGG ATG GCG CAC GCT GGG AGA ACG
M A H A G R T

1530
*
GGG TAC GAC AAC CGG GAG ATA GTG ATG AAG TAC ATC CAT TAT AAG CTG TCG
G Y D N R E I V M K Y I H Y K L S

1581
*
CAG AGG GCC TAC GAG TGG GAT GCG GGA GAT GTG GCG GCC GCG CCC CCG GGG
Q R G Y E W D A G D V G A A P P G

1632
*
GCC GCC CCC GCA CCG GGC ATC TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT
A A P A P G I F S S Q P G H T P H

1683
*
CCA GCC GCA TCC CGC GAC CCG GTC GCG ACC AGG ACC TCG CCG CTG CAG ACC CCG
P A A S R D P V A R T S P L Q T P

1734
*
GCT GCC CCC GGC GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT GTG
A A P G A A A G P A L S P V P P V

Fig. 13

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1785
 *
 GTC CAC CTG GCC CTC CGC CAA GCC GGC GAC GAC TTC TCC CGC CGC TAC CGC
 V H L A L R Q A G D D F S R R Y R

1836
 *
 GGC GAC TTC GCC GAG ATG TCC AGC CAG CTG CAC CTG ACG CCC TTC ACC GCG
 G D F A E B M S S Q L H L T P F T A

1887
 *
 CGG GGA CGC TTT GCC ACG GTG GAG GAG CTC TTC AGG GAC GGC GTG AAC
 R G R P A T V V E B L F R D G V N

1938
 *
 TGG GGG AGG ATT GTG GCC TTC TTT GAG TTC GGT GGC GTC ATG TGT GTG GAG
 W G R I V A F F E B F G G V M C V E

1989
 *
 AGC GTC AAC CGG GAG ATG TCG CCC CTG GGC GAC AAC ATC GCC CTG TGG ATG
 S V N R E B M S P L V D N I A L W M

2040
 *
 ACT GAG TAC CTG AAC CGG CAC CTG CAC ACC TGG ATC CAG GAT AAC GGA GGC
 T B Y L N R H L H T W I Q D N G G

Fig. 13

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TGG GAT GCC TTT GTG GAA CTG TAC GGC CCC AGC ATG CGG CCT CTG TTT GAT
 W D A F V E L Y G P S H R P L F D

2142

TTC TCC TGG CTG TCT CTG AAG ACT CTG CTC CTG GCC CTG GTG GGA GCT
 F S W L S L X T L L S L A L V G A

2193

TGC ATC ACC CTG GGT GCC TAT CTG AGC CAC AAG TGA AGT CAA CAT GCC TGC
 C I T L G A Y L S H K *

Fig. 13

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Viability of Cells at Maximum Density

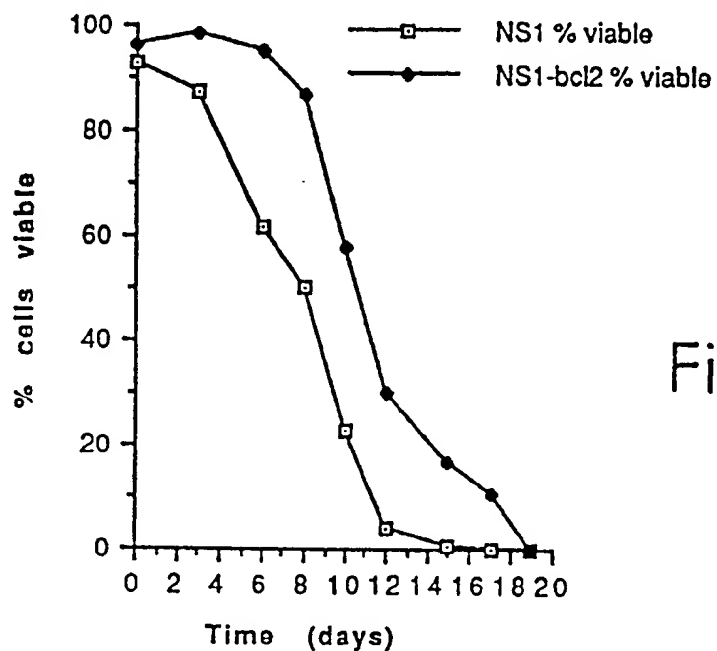


Fig. 14A

Cell Numbers at Maximum Density

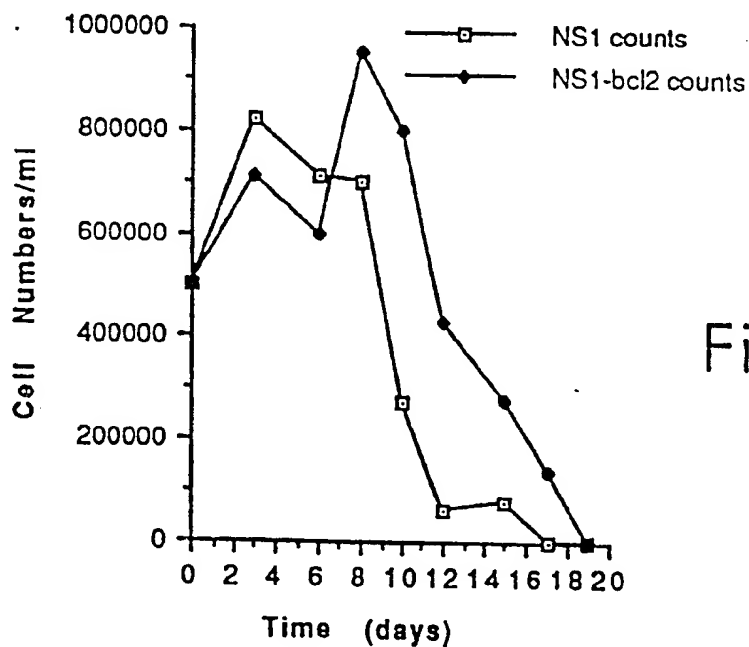


Fig. 14B

NS1 survival in 0.5% Foetal Calf Serum (FCS)

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Cell Numbers in 0.5% FCS

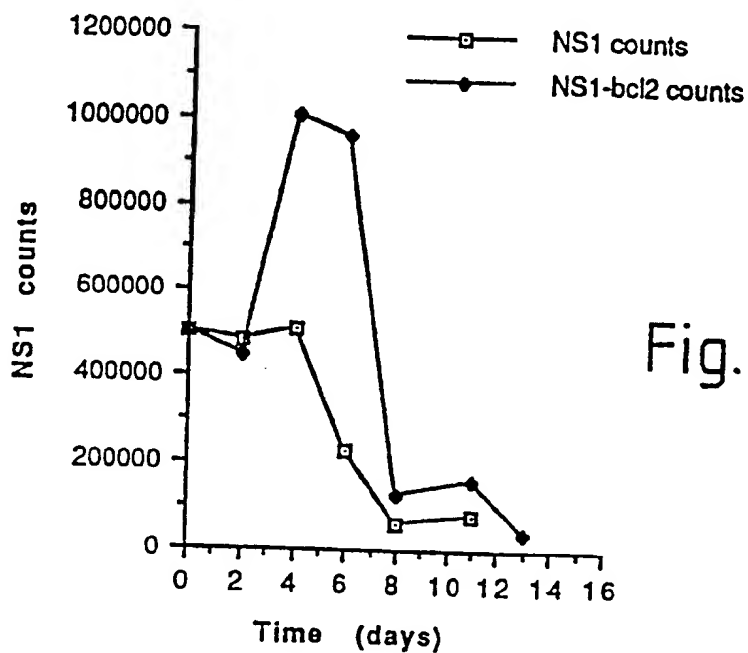


Fig. 15A

%age cells viable in 0.5% FCS

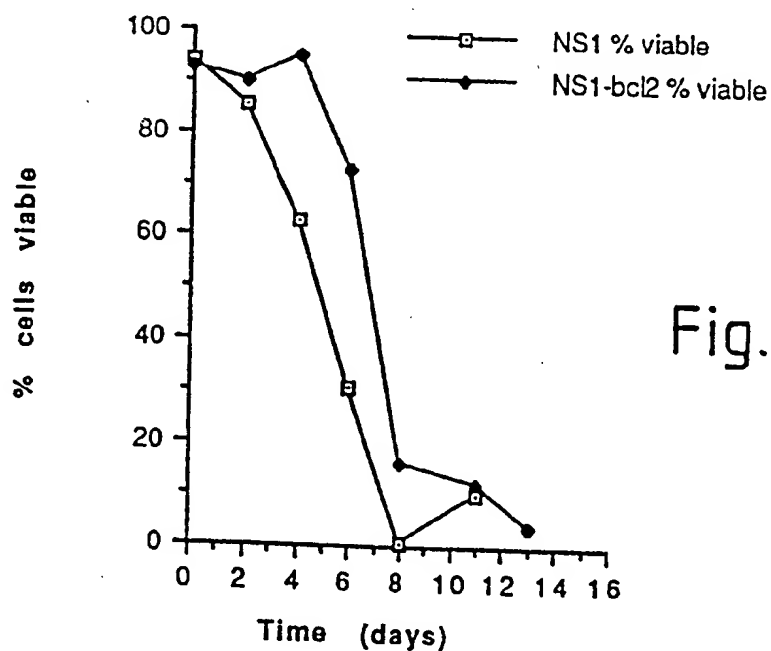


Fig. 15B

INTERNATIONAL SEARCH REPORT

PCT/GB 93/00686

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5	C12N15/12; C12Q1/68;	C12N15/11; C12N5/10;
	A61K37/02; C12N15/62	A61K31/70
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; C12N ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 87, May 1990, WASHINGTON US pages 3660 - 3664 REED, J.C. ET AL. 'BCL2-mediated tumorigenicity of a human T-lymphoid cell line: synergy with MYC and inhibition by BCL2 antisense' see the whole document	1,4-6,8, 26
Y	---	7
	-/--	
¹⁰ Special categories of cited documents: ¹⁰ ^{"A"} document defining the general state of the art which is not considered to be of particular relevance ^{"E"} earlier document but published on or after the international filing date ^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) ^{"O"} document referring to an oral disclosure, use, exhibition or other means ^{"P"} document published prior to the international filing date but later than the priority date claimed ^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention ^{"X"} document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step ^{"Y"} document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art ^{"A"} document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
27 JULY 1993	12. 08. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	ANDRES S.M.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>CELL vol. 67, 29 November 1991, CAMBRIDGE, NA US pages 889 - 899 STRASSER, A. ET AL. 'bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship' cited in the application</p>	26
Y	<p>see page 890, right column: 'Survival and differentiation of T lymphoid cells in vitro' see page 897, left column, paragraph 3 ---</p>	1-6,8,26
Y	<p>MOLECULAR AND CELLULAR BIOLOGY vol. 10, no. 8, August 1990, WASHINGTON US pages 4370 - 4374 REED, J.C. ET AL. 'Complementation by BCL2 and C-HA-RAS oncogenes in malignant transformation of rat embryo fibroblasts' cited in the application see the whole document ---</p>	1-6,8,26
X	<p>NATURE vol. 348, 22 November 1990, LONDON GB pages 334 - 336 HOCKENBERRY, D. ET AL. 'Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death' cited in the application see the whole document ---</p>	26
Y	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 83, March 1986, WASHINGTON US pages 1227 - 1231 TOULMÉ, J.J. ET AL. 'Specific inhibition of mRNA translation by complementary oligonucleotides covalently linked to intercalating agents' see the whole document ---</p>	7
A	<p>NATURE vol. 340, 6 July 1989, LONDON GB pages 66 - 68 EILERS, M. ET AL. 'Chimaeras of Myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells' cited in the application see the whole document -----</p>	30

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB93/00686

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-8 are directed to a method of treatment of the human or animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see annex
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

In his FAX DATED FROM April 7, 1993, the Applicant requested the search being done on the set of claims as originally filed, i.e. claims 1-8 and 24-30.

The Search Authority has found claims 24 and 25 to be unsearchable. Indeed, claim 24 defines a specific embodiment of a method which it refers to be described in the (for the Search Authority) non-existent claim 23, and claim 25 refers to products prepared by a method according to claims 22-24. Therefore, no search has been done for claims 24 and 25.

